Halothane Binding to a G Protein Coupled Receptor in Retinal Membranes by Photoaffinity Labeling†

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ABSTRACT: General anesthetics have been reported to alter the functions of G protein coupled receptor (GPCR) signaling systems. To determine whether these effects might be mediated by direct binding interactions with the GPCR or its associated G protein, we studied the binding character of halothane on mammalian rhodopsin, structurally the best understood GPCR, by using direct photoaffinity labeling with [^{14}C]halothane. In the bleached bovine rod disk membranes (RDM), opsin and membrane lipids were dominantly photolabeled with [^{14}C]halothane, but none of the three G protein subunits were labeled. In opsin itself, halothane labeling was inhibited by unlabeled halothane with an IC_{50} of 0.9 mM and a Hill coefficient of −0.8. The stoichiometry was 1.1:1.0 (halothane:opsin molar ratio). The IC_{50} values of isoflurane and 1-chloro-1,2,2-trifluorocyclobutane were 5.0 and 15 mM, respectively. Ethanol had no effect on opsin labeling by halothane. A nonimmobilizer, 1,2-dichlorohexafluorocyclobutane, inhibited halothane labeling by 50% at 0.05 mM. The present results demonstrate that halothane binds specifically and selectively to GPCRs in the RDM. The absence of halothane binding to any of the G protein subunits strongly suggests that the functional effects of halothane on GPCR signaling systems are mediated by direct interactions with receptor proteins.

General anesthetics have been considered to act on neuronal membranes, and exert their effects on synaptic transmission (*1*). Neuronal membrane proteins, including a variety of receptors and channels, are responsible for information transfer across the synaptic membranes, and have therefore attracted attention as potentially relevant anesthetic targets. The activity of many neuronal membrane proteins, such as the ligand-gated ion channels, has been demonstrated to be sensitive to anesthetics (*1*), but the molecular interactions underlying the changes in activity are poorly understood. Indeed, it remains unclear whether anesthetics exert their effect through direct binding interactions with the protein, or whether they simply alter important properties of membrane lipids.

G protein coupled receptors (GPCRs) are neuronal membrane proteins that recognize and transmit signals from diverse ligands to a variety of G proteins, which in turn amplify and sustain the duration of the signals (*2*). Not only in brain function, but also in hormonal control of cardiovascular system and metabolism, GPCRs and G proteins are essential signaling intermediaries. The extensive list of neurotransmitters and neuromodulators that work through GPCRs includes acetylcholine, noradrenaline, dopamine, adenosine, and opioids (*3*). The functional significance of the effects of volatile anesthetics on these GPCRs has been shown in several different in vitro systems. Halothane inhibited m1 muscarinic (*4*) as well as thromboxane A_2 receptor signaling in *Xenopus* oocytes (*5*). Halothane was also shown to reduce the agonist binding affinity of the β-adrenergic receptor in rat myocardium (*6*). These data agree that the site of halothane action is most likely the receptor and/or its associated G protein, but not intracellular signaling systems. In addition, these data suggest that widely distributed GPCR signaling systems might be targets that contribute to anesthetic action. However, direct binding of any volatile anesthetic to GPCRs or G protein has not yet been demonstrated.

Mammalian rhodopsin is a member of the 7-helix GPCR family. Structure−function relationships in rhodopsin have been extensively studied in order to understand the molecular mechanisms of signal transduction by GPCRs in general (*2*, *3*). Although rhodopsin is a photoreceptor and we are not proposing rhodopsin itself as an important anesthetic target, rhodopsin is considered a reasonable model of the GPCRs of perhaps greater relevance to anesthesia for the following reasons: (1) The primary to tertiary structural features of the rhodopsin family of GPCRs, which includes many neurotransmitter receptors discussed above, are highly conserved (*3*). (2) Ligand binding sites in the transmembrane core in these GPCRs are similar, particularly the sites for biogenic amines and rhodopsin ligand retinal (*2*, *7*, *8*). (3) G protein binding domains are highly homologous (*7*). (4) Transducin (Gt), the G protein associated with rhodopsin, is most similar to Gi. Gt α subunit has approximately 70%

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amino acid homology to Gi (*9*). In addition to these substantial similarities, functional effects of volatile anesthetics on rhodopsin have been demonstrated in both in vivo (*10*) and in vitro systems (*11, 12*). Finally, among these GPCRs, rhodopsin is the only one that can be obtained in high mass and purity in native biological membranes (*13*), an important practical consideration when attempting to relate weak binding to a single molecular target. Therefore, confirming and characterizing binding of halothane to rhodopsin in the retinal membranes could give us significant insight into the molecular interactions of anesthetics with GPCRs.

METHODS

Materials. Fresh bovine eyes were obtained from a local slaughterhouse (MOPAC, Inc., Soudertown, PA), and the retinas were dissected in room light. Rod disk membranes (RDM)[1] were prepared by sucrose flotation in isotonic buffer (20 mM MOPS, 100 mM KCl, 6 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mM EDTA, pH 7) as previously described (*14*). The membranes were stored in the same medium on ice at a rhodopsin concentration of 200−300 μM under argon. Absorption spectra of the RDM measured before experiments showed no specific peak for rhodopsin conformers. [^{14}C]Halothane (2-bromo-2-chloro-1,1,1-[1-^{14}C]-trifluoroethane) (specific activity 51 mCi/mmol) was purchased from DuPont, NEN (Boston, MA). Labeled halothane was further purified by HPLC on a C4 column (Supelcosil, LC-304, SUPELCO, Bellefonte, PA) using a solvent system of H_2O−methanol (4:1). All final photolabeling solutions contained less than 0.5 M methanol. We thus tested photoaffinity labeling of [^{14}C]halothane to RDM with increasing concentrations of methanol, 30 mM−3.0 M. Methanol did not show significant displacement of halothane labeling up to the concentration of 1.0 M. Unlabeled halothane was obtained from Halocarbon Laboratories (Hackensack, NJ). The thymol preservative was removed before use with an aluminum oxide column. Isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether) was purchased from Ohmeda PPD, Inc. (Liberty Corner, NJ). 1-Chloro-1,2,2-trifluorocyclobutane (F3) and 1,2-dichlorohexafluorocyclobutane (F6) were obtained from PCR Incorporated (Gainesville, FL). All other chemicals were reagent grade and were purchased from Sigma Chemical Co. (St. Louis, MO).

Photoaffinity Labeling of Halothane to Retinal Membranes. RDM were incubated with [^{14}C]halothane in isotonic MOPS buffer in 2 mL quartz cuvettes (5 mm path length) at room temperature in the light and were exposed to 254 nm light (Hg pencil calibration lamp; Oriel, Stanford, CT) at a distance of ~5 mm for 60 s with continuous stirring. The final concentrations in the photolabeling solution were opsin 25 μM and [^{14}C]halothane 16 μM. The photolabeled RDM were centrifuged and washed twice with isotonic MOPS buffer. G proteins were then removed from labeled retinal membranes by washing 3 times with 100 μM GTP in hypotonic buffer (10 mM MOPS, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, pH 7) as previously described (*15*). Supernatant was collected and microconcentrated using 10 kDa molecular weight cutoff membranes (Microcon10, Amicon Inc., Beverly, MA). SDS/PAGE of the labeled membranes and isolated G proteins was performed in modified Laemmli gels [acrylamide 15%, bisacrylamide 0.03%, tris(hydroxymethyl)aminomethane 380 mM, and SDS 0.1%], and followed by autoradiography of the dried gel.

Protection from [^{14}C]Halothane Labeling by Unlabeled Halothane. RDM were incubated with [^{14}C]halothane and with increasing concentrations of unlabeled halothane in potassium phosphate buffer (20 mM KH_2PO_4, 2 mM $MgCl_2$, 100 mM KCl, pH 7) in 2 mL quartz cuvettes at room temperature in the light and were exposed to 254 nm light as described above. The final concentrations in the photolabeling solution were opsin 3.1 μM, [^{14}C]halothane 3.4 ± 0.7 μM, and unlabeled halothane 0−6.0 mM. One milliliter of the cuvette contents was vacuum-filtered through glass microfiber filters (GF/B, Whatman International Ltd., England) and washed with 15 mL of ice-cold saline. Retained label on the filters was counted by liquid scintillation, which we defined as label incorporation in the RDM. SDS/PAGE of the remaining labeled membranes was performed in the modified Laemmli gels, and the gel was stained with Coomassie blue. The relative mass of opsin in the bands on SDS/PAGE was determined by reflective density using an imaging densitometer (GS-710, Bio-Rad Laboratories, Hercules, CA). Rhodopsin bands in the dried gels were excised and dissolved by incubating with 30% hydrogen peroxide at 60 °C for 5 h. Label incorporation into opsin itself was determined in the dissolved gel slices by scintillation counting, and disintegrations per minute (dpm) were normalized to the relative mass of opsin. We defined this dpm as label incorporation in opsin.

Protection from [^{14}C]Halothane Labeling by Other Anesthetics and a Nonimmobilizer. Photoaffinity labeling of [^{14}C]halothane to the RDM was evaluated in the presence of isoflurane, F3, F6, and ethanol at the concentrations given in the figures. Isoflurane, F3, and F6 were prepared in potassium phosphate buffer, and allowed to equilibrate for several hours after vigorous vortexing and sonication. Photoaffinity labeling and SDS/PAGE were performed following the protocol described above.

Quantitation and Statistics. The data of the halothane protection study were fitted to sigmoid dose−response curves with variable slopes using nonlinear least-squares regression. The IC_{50} value is the concentration of the compound required to inhibit half of the inhibitable [^{14}C]halothane labeling. The bottom of the protection curve was forced to zero for F6 and ethanol in RDM, and halothane and F3 in opsin, because the initial fitted values were negative.

RESULTS

Photoaffinity Labeling of Halothane to Bovine Retinal Membranes. SDS/PAGE of the RDM and the isolated G proteins and their resulting autoradiograms are shown in Figure 1. Photolabeling of the RDM with [^{14}C]halothane resulted in dominant label incorporation into opsin and also into membrane lipids. Scintillation counting of the dissolved gel slices confirmed that 43 ± 1% (3 gels) of the total label in the RDM was found in the rhodopsin band and 52 ± 2%

[1] Abbreviations: RDM, rod disk membranes; F3, 1-chloro-1,2,2-trifluorocyclobutane; F6, 1,2-dichlorohexafluorocyclobutane; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; MOPS, morpholinopropanesulfonic acid; EDTA, ethylenedinitrilotetraacetic acid.

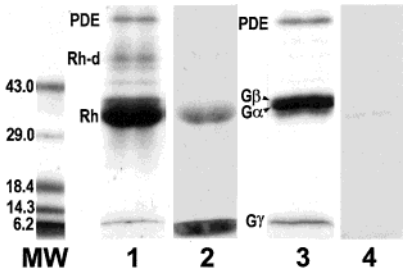

FIGURE 1: SDS/PAGE of the retinal membranes (lane 1) and the resulting autoradiogram (lane 2), and SDS/PAGE of the isolated G protein (lane 3) and its autoradiogram (lane 4). Lanes 1 and 3 are stained with Coomassie blue. Lane MW is size markers, which from the top are ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa), and bovine trypsin inhibitor (6.2 kDa) (Life Technologies). The prominent bands in lane 1 from the top are phosphodiesterase (PDE), rhodopsin (opsin) dimer (Rh-d), and rhodopsin (opsin) monomer (Rh). The two small bands immediately above the rhodopsin monomer are G protein β and α subunits. The resulting autoradiogram (lane 2) shows dominant incorporation into opsin and lipids (below the 6.2 kDa standard). In lane 3, the prominent bands from the top are phosphodiesterase (PDE), G protein β subunit (Gβ), α subunit (Gα), and γ subunit (Gγ). The weak band immediately below the α subunit is rhodopsin. There is no appreciable label incorporation in G protein subunits in the autoradiogram (lane 4).

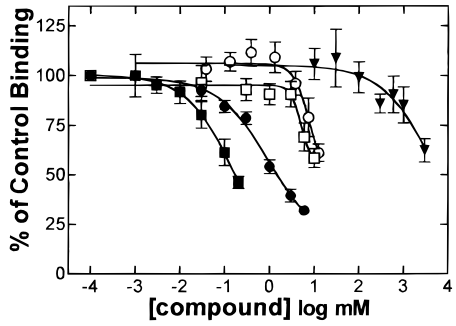

FIGURE 2: Protection from [^{14}C]halothane photoaffinity labeling by four different anesthetics and a nonimmobilizer in the retinal membranes. Control binding is that in the absence of any compound. Points are normalized to the control binding, and represent 7 separate experiments for halothane and 6 separate experiments for the other compounds. The data are fitted to sigmoidal dose−response curves (variable slopes) with nonlinear least-squares regression. The IC_{50} values are shown in Table 1. ● = halothane; ○ = isoflurane; □ = 1-chloro-1,2,2-trifluorocyclobutane (F3); ■ = 1,2-dichlorohexafluorocyclobutane (F6); ▼ = ethanol. Values are means ± SEM.

in the lipids. The autoradiogram showed that label incorporation into any of the G protein subunits was not appreciable. This result was confirmed by scintillation counting of the dissolved gel slices of the G protein α, β, and γ subunits. Because of the possibility that halothane binds to receptor-unbound G proteins, we studied photoaffinity labeling of [^{14}C]halothane to purified G proteins in the same condition as described above. Label incorporation into the purified G proteins was at least 10-fold lower than that in rhodopsin.

Protection from Halothane Labeling with Halothane, Isoflurane, F3, F6, and Ethanol. Figure 2 illustrates protection from halothane photolabeling in the RDM by anesthetics and a nonimmobilizer. With increasing concentrations of unlabeled halothane, 85% of the [^{14}C]halothane labeling was inhibitable with an IC_{50} of 0.9 mM and a Hill coefficient of −0.8. At the IC_{50} of halothane, 5.2 ± 1.3 nmol of halothane (mean ± SEM, n = 7) was incorporated into the RDM containing 3.1 nmol of opsin. Incorporation of [^{14}C]halothane into the RDM was significantly inhibited in the presence of F6, F3, isoflurane, and ethanol. F3, isoflurane, and ethanol inhibited halothane labeling at higher concentrations with IC_{50} values of 5.1 mM, 8.1 mM, and 5.2 M, respectively (Table 1). F6, a nonimmobilizer, displaced halothane binding by 50% at a concentration of 0.17 mM.

Figure 3 illustrates protection from halothane photolabeling in opsin itself (from excised, dissolved gel bands). Label incorporation to opsin in SDS/PAGE is presented as dpm normalized to the relative mass of opsin in the bands using reflective density. Incorporation of [^{14}C]halothane into opsin was significantly inhibited by unlabeled halothane with an IC_{50} value of 0.9 mM and a Hill coefficient of −0.8. At the IC_{50} of halothane, 0.53 ± 0.01 nmol of halothane was incorporated into opsin 1.00 ± 0.03 nmol (mean ± SEM, n = 7). IC_{50} values of isoflurane and F3 were 5.0 and 15 mM, respectively (Table 1). Incorporation of [^{14}C]halothane into opsin was not inhibited by ethanol. F6 inhibited halothane photolabeling in opsin with an IC_{50} of 0.05 mM. To determine stoichiometry, we used label incorporated in the rhodopsin band in SDS/PAGE. Stoichiometry based on halothane incorporation at IC_{50} is 0.53 ± 0.02 (halothane: opsin molar ratio). The maximum stoichiometry is thus 1.1 (halothane/opsin).

Figure 4 shows a gradual decrease in label incorporation in opsin in the SDS/PAGE autoradiogram with increasing concentrations of unlabeled halothane. The relative mass of opsin in the SDS/PAGE decreased at high concentrations of halothane, indicating the predictable aggregation/cross-linking of opsin by ultraviolet light (UV).

DISCUSSION

Stoichiometry for Halothane in Rhodopsin. The present results demonstrate preferential binding of halothane to GPCRs in bovine retinal membranes in the low millimolar concentration range. Halothane was incorporated into opsin with the calculated stoichiometry of 1.1:1 (halothane: rhodopsin), probably indicating only a single specific binding site for halothane in this 40 kDa receptor. For comparison, the nicotinic acetylcholine receptor (290 kDa) in *Torpedo* membranes was photolabeled with [^{14}C]halothane with at least a 5:1 halothane:receptor molar ratio (*16*). Skeletal muscle Ca^{2+}-ATPase (110 kDa) was also labeled with a 5:1 stoichiometry, a value we recalculated based on the published data (*17*). Thus, the stoichiometry of halothane labeling in these membrane proteins is not a simple feature of molecular mass, but appears to rely on the different tertiary and quaternary structures of these proteins. This finding is also consistent with our previous finding in soluble proteins that specific halothane binding is a feature of the tertiary structure of the proteins (*18*, *19*). In addition, binding stoichiometry may be dependent on more subtle conformational shifts. Rhodopsin is known to undergo a series of light-induced conformational changes, during which helix orientation as well as receptor volume are reported to change (*2*). Potential anesthetic binding sites, such as the hydrophobic core or a lipid−protein interface, might also change during the cycle. The present experiments were performed only on bleached rhodopsin, opsin, in the RDM, which is the receptor protein without bound ligand. Therefore, further work will be required to isolate and label the various rhodopsin conformers.

Table 1: IC_{50} of Anesthetics and a Nonimmobilizer for Protection from Halothane Photolabeling in the Retinal Membranes and in Opsin

	RDM			opsin			
agent	IC_{50} (mM)	95% CI of IC_{50} (mM)	maximum protection (%)[a]	IC_{50} (mM)	95% CI of IC_{50} (mM)	maximum protection (%)[a]	EC_{50} (mM)
halothane	0.9	0.2−4.7	85 ± 13	0.9	0.7−1.3	100	0.23[b]
isoflurane	8.1	1.7−38.1	55 ± 33	5.0	1.0−24	49 ± 15	0.29[c]
F6	0.17	0.15−0.19	100	0.05	0.01−0.23	90 ± 17	−
F3	5.1	2.9−8.8	44 ± 7	15.3	5.5−42.5	100	1.47[d]
ethanol	5240	2000−13700	100	−	−	0	190[e]

[a] The values of maximum protection were obtained from the best fitted sigmoidal dose−response curves using nonlinear least-squares regression. The bottoms of the protection curves were forced to zero for F6 and ethanol in RDM, and halothane and F3 in opsin, because the initial fitted values were negative. Values are means ± SEM. [b] EC_{50} value was determined in tadpoles at 25 °C (*21*). [c] EC_{50} in tadpoles (temperature not indicated) (*22*). [d] EC_{50} in rats (*20*). [e] EC_{50} in tadpoles at 20 °C (*43*).

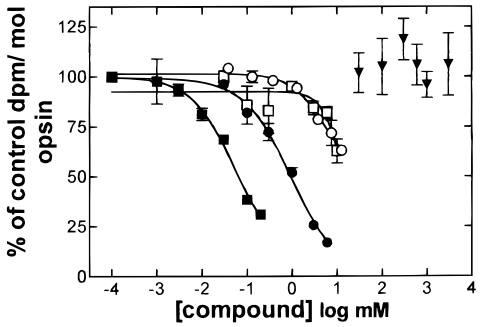

FIGURE 3: Protection from [^{14}C]halothane photoaffinity labeling by different anesthetics and a nonimmobilizer in the isolated opsin. Label incorporation in opsin is presented in disintegrations per minute (dpm), and the amount of opsin in the bands is corrected by reflective density. Control dpm/mol opsin is that in the absence of any compound, and points are normalized to the control. The data are fitted to sigmoidal dose−response curves (variable slopes) with nonlinear least-squares regression. The IC_{50} values are shown in Table 1. ● = halothane; ○ = isoflurane; □ = 1-chloro-1,2,2-trifluorocyclobutane (F3); ■ = 1,2-dichlorohexafluorocyclobutane (F6); ▼ = ethanol. Values are means ± SEM.

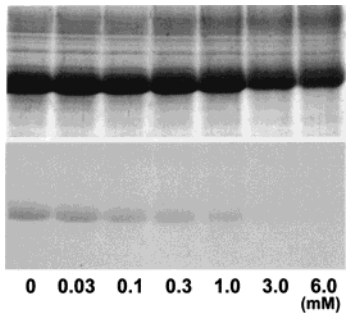

FIGURE 4: SDS/PAGE (upper panel) and the resulting autoradiogram (lower panel) for protection from [^{14}C]halothane photoaffinity labeling by increasing concentrations of unlabeled halothane. SDS/PAGE are stained with Coomassie blue. Unlabeled halothane concentrations are shown under the autoradiogram. The relative mass of opsin in the SDS/PAGE significantly decreased to 80 ± 3% and 69 ± 2% at halothane concentrations of 3.0 and 6.0 mM, respectively, compared to the opsin without unlabeled halothane.

Specificity and Selectivity of Halothane Binding in Rhodopsin. Considerable differences in the ability to inhibit halothane labeling between the other anesthetics strongly suggest that binding sites for different anesthetics are distinct. In addition, the binding sites for halothane in opsin appear to be fairly specific. The IC_{50} of halothane in opsin was 0.9 mM, only 2-fold higher than the immobilization EC_{50} value previously reported in rats (*20*) and 4-fold higher than the analogous EC_{50} value in tadpoles (*21*). On the other hand, the IC_{50} of isoflurane was at least 10-fold higher than its EC_{50} values (*20*, *22*), and ethanol did not provide significant protection from halothane photolabeling in opsin. The potencies of these anesthetics and the nonimmobilizer, except ethanol, to inhibit halothane labeling in opsin are similar to those in the intact RDM, suggesting the specific binding sites for halothane in the membranes reside in rhodopsin.

In the present study, ethanol was unique among the anesthetics used. Ethanol inhibited halothane labeling in the RDM only at very high concentrations, but did not affect halothane labeling in opsin, suggesting the ethanol-inhibitable fraction in the RDM is lipid. Consistent with this interpretation, ethanol has been shown to interact with specific domains of membrane lipids, and the lipid−protein interface appears to be one of the sites affected by ethanol (*23*). It is thus possible that specific interactions of ethanol with membrane lipids could displace halothane labeling in the RDM lipids. In this context, it is of interest that ethanol is thought to affect some membrane protein functions through lipid-mediated mechanisms. For example, ethanol enhanced metarhodopsin II formation by disordering phospholipid acyl chain packing in the RDM (*24*). Similarly, another study showed that the effect of ethanol on protein kinese C (PKC) activity was dependent on the lipid composition of the bilayers (*25*). However, we cannot entirely eliminate the possibility that ethanol directly interacts with rhodopsin. Direct interactions of ethanol with membrane-associated proteins have been demonstrated in PKC (*25*), and saturable ethanol binding to membranes (*26*) also suggests interactions with protein. The present result indicates that, if there is an ethanol binding site in rhodopsin, it is expected to have little communication with the halothane binding sites.

A nonimmobilizer, F6, protected opsin from halothane photolabeling with an IC_{50} of 0.05 mM, suggesting that F6 shares the binding domain with halothane in opsin. Although nonimmobilizers, or nonanesthetics, do not prevent animals from moving in response to noxious stimuli at concentrations predicted to be anesthetic based on lipid-partitioning (*27*), F6 has been reported to bind in protein sites where anesthetics do. F6 quenched human serum albumin (HSA) fluorescence from its single tryptophan with a dissociation constant of 160 μM (*28*), indicating that F6 binds in the same HSA domain as halothane (*29*, *30*). However, the effects of F6 on global stability and regional dynamics of the soluble proteins are opposite to anesthetics (*31*, *32*). Further, it has been reported that F6, as well as halothane, F3, and ethanol, inhibited muscarinic m1 (*33*) and 5-HT type 2A receptor induced currents in *Xenopus* oocytes (*34*). However, a PKC

inhibitor abolished the effects of the anesthetics on both receptors, but had no effect on F6 actions, suggesting the inhibitory mechanism of F6 is different from halothane and does not involve PKC. In addition, a recent report suggested F6 affects learning and memory (*35*), in which muscarinic receptors are considered to play an important role. Taken together, these data suggest that F6 may exhibit a form of isosteric competition, a commonly accepted mechanism of many receptor antagonists.

Volatile Anesthetics and GPCR Signaling System. The present results clearly show the absence of halothane photolabeling to any of G protein subunits in the RDM. This suggests that functional effects of halothane on the rhodopsin signaling system are mediated through direct interactions with the receptor protein. Although the present study was not designed to address the functional influence of halothane on rhodopsin, specific binding can be associated with specific functional changes of protein (*36*). In fact, volatile anesthetics and alcohol have been reported to affect conformational changes in rhodopsin. Halothane was shown to reduce the light-induced proton uptake of rhodopsin in RDM (*11*), suggesting that halothane may inhibit the metarhodopsin I to metarhodopsin II transition. This effect was also illustrated by enflurane (*12*) and ethanol (*24*) using absorption spectra. Furthermore, dark adaptation, in which rhodopsin regeneration with retinal from opsin plays an important role, was retarded in humans under halothane anesthesia (*10*). These findings suggest that anesthetics may preferentially bind to a certain conformer of rhodopsin. Considerable conformational dependence of halothane photolabeling has been demonstrated in soluble protein (*36*). Photoaffinity labeling on regenerated rhodopsin and its conformers could give us more insight for functional effects of halothane binding in GPCRs.

A number of studies have shown that volatile anesthetics interfere with other GPCR signaling as well. Halothane has been reported to inhibit thromboxane A_2 signaling in a competitive way at the membrane receptor (*5, 37*). Halothane, however, has no effect on intracellular signaling pathways in this system, indicating that halothane interacts with the receptor or receptor−G protein coupling (*5*). This effect was also demonstrated in m1 muscarinic receptor signaling (*4*). In addition, halothane decreased ligand binding affinity in opioid receptor from guinea-pig brain (*38*) and in β-adrenergic receptor in rat myocardium (*6*). Although these data clearly show a wide spectrum of anesthetic effects through GPCR signaling systems, differentiation of possible anesthetic binding sites, such as between the receptor and its associated G protein, does not appear to be detectable in the experimental settings used in these studies. Therefore, further identification of anesthetic binding sites in this type of receptor needs to be done with the use of combined experimental techniques including photoaffinity labeling.

Lipid Photolabeling in the RDM. The present study shows that some of the inhibitable labeling can be attributed to membrane lipids. In the RDM, approximately 85% of the halothane labeling was inhibited by unlabeled halothane (Figure 2), which is greater than the total label incorporation into opsin itself. Although volatile anesthetic partitioning into membrane lipids has long been considered to be nonspecific and nonsaturable, a recent NMR spectroscopic study shows xenon interacts saturably and preferentially with the amphiphilic head region of a model membrane (*39*). Further, recent molecular dynamics studies show that anesthetic molecules partition into localized regions within the lipid bilayers (*40*). These data indicate anesthetics may interact specifically with the distinct structural organization of lipid bilayers, suggesting saturability, and encourage future studies on preferential localization of halothane in the RDM lipids for a greater understanding of halothane dynamics in biological membranes.

Halothane and other volatile anesthetics are known to partition well into the lipid bilayers. The synaptosomal membrane/buffer partition coefficient for halothane was reported to be 27.5 (*41*), and phosphatidylcholine:cholesterol (2:1) bilayer/buffer partitioning was 50 (*42*). One could thus argue that preferential binding of halothane to rhodopsin, but not to G protein, might be only due to a higher concentration of halothane in the RDM than in buffer. However, thermodynamics dictate similar occupancy of otherwise similar protein sites in different environments when referenced to the same standard state (in this case, water). In other words, preferential partitioning of halothane into lipids as compared to water indicates lower binding constants for the same protein site when immersed in lipids (smaller free energy difference for anesthetic·lipid−anesthetic·protein than for anesthetic·water−anesthetic·protein). The lower binding constants are compensated by the higher concentration, and occupancy becomes equivalent. In addition, halothane labels the 65 kDa bovine serum albumin (BSA) with a stoichiometry of ∼5:1 (halothane:protein molar ratio) (*36*), far higher than the stoichiometry for rhodopsin in RDM at a similar aqueous halothane concentration. Therefore, both theory and data indicate that halothane concentration differences between buffer and membranes do not explain preferential binding to rhodopsin as compared to transducin. The lack of transducin labeling is due to the lack of specific binding sites, suggesting that any effects of halothane on this protein are indirect.

Limitations. Rapid binding kinetics of small molecules such as volatile anesthetics preclude conventional binding studies. We thus used direct halothane photoaffinity labeling to characterize halothane binding in the present study. However, the photochemistry involved in the direct photoaffinity labeling technique is complex and not completely characterized (*18*). UV light exposure converts the rapid kinetics of halothane equilibrium binding to covalent linkage by creating a reactive chlorotrifluoroethyl radical. Accordingly, the labeling produced by UV exposure for 30 or 60 s in the present study may not necessarily reflect halothane equilibrium binding. However, close agreement between equilibrium and photolabeling approaches has been obtained in several soluble proteins (*29, 30*). Membrane proteins represent a more complex system, in which features of potential anesthetic binding sites, such as a lipid−protein interface, may be fundamentally different from those in soluble proteins. Thus, anesthetic binding sites in rhodopsin in the RDM need to be further studied using equilibrium binding techniques.

In summary, the present data clearly show specific and selective binding of halothane to GPCRs, but not to the associated G protein. To allow extrapolation to other GPCRs in the CNS, further characterization of the binding sites and functional effects is required.

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