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G Protein-Coupled Receptors as Direct Targets of Inhaled Anesthetics

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ABSTRACT
The molecular pharmacology of inhalational anesthetics remains poorly understood. Despite accumulating evidence suggesting that neuronal membrane proteins are potential targets of inhaled anesthetics, most currently favored membrane protein targets lack any direct evidence for anesthetic binding. We report herein the location of the binding site for the inhaled anesthetic halothane at the amino acid residue level of resolution in the ligand binding cavity in a prototypical G protein-coupled receptor, bovine rhodopsin. Tryptophan fluorescence quenching and direct photoaffinity labeling with [14C]halothane suggested an interhelical location of halothane with a stoichiometry of 1 (halothane/rhodopsin molar ratio). Radiosequence analysis of [14C]halothane-labeled rhodopsin revealed that halothane contacts an amino acid residue (Trp265) lining the ligand binding cavity in the transmembrane core of the receptor. The predicted functional consequence, competition between halothane and the ligand retinal, was shown here by spectroscopy and is known to exist in vivo. These data suggest that competition with endogenous ligands may be a general mechanism of the action of halothane at this large family of signaling proteins.

The mechanisms of general anesthetic action at the molecular level remain poorly understood, despite their use in millions of patients each year. Understanding the molecular mechanisms by which inhaled anesthetics produce behavioral effects, such as loss of consciousness and analgesia, is thus an important goal with therapeutic implications. Accumulating evidence suggests that these drugs act at multiple neuronal membrane proteins that function as ion channels and neurotransmitter receptors (Franks and Lieb, 1994). However, classification as an anesthetic target requires evidence of direct binding, and most currently favored targets lack any direct evidence for anesthetic binding. One of the major difficulties in demonstrating direct binding is the weak binding energetics of the inhaled anesthetic (Eckenhoff and Johansson, 1997). Weak binding, although consistent with the relatively featureless molecules and the high aqueous EC50 for general anesthesia in mammals (0.2–1.0 mM) (Franks and Lieb, 1994), essentially precludes conventional radioligand binding studies. Furthermore, there have been few good model systems for studying the actions of inhaled anesthetics in biological membranes (Forman et al., 1997). In addition to plausible roles in central nervous system signaling, it is important for potential model proteins to be available in sufficient abundance and purity to permit direct binding and high-resolution structural studies.

A large superfamily of G protein-coupled receptors (GPCRs) modulates most signaling in central and peripheral nervous systems. In particular, the rhodopsin family of GPCRs includes many neurotransmitter receptors, such as muscarinic acetylcholine, noradrenaline, dopamine, adenosine, and opioid receptors (Baldwin et al., 1997). These receptors have highly conserved regions in the transmembrane portion (Baldwin et al., 1997), and the ligand-receptor interactions in the core formed by the seven α-helices are thought to be similar in the GPCRs of this family (Strader et al., 1994; Ji et al., 1998). Functionally, cholinergic neurotransmission is known to influence awareness, sleep, and learning and memory (Durieux, 1996). The α2-adrenergic receptor seems to play a role in antinociceptive responses as well as in the

ABBREVIATIONS: GPCR, G protein-coupled receptor; RDM, rod disk membranes; MOPS, 3-(N-morpholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin; NATA, N-acetyl-tryptophan-amide; PKC, protein kinase C.
state of arousal (Bol et al., 1999). In fact, agonists and/or antagonists that work through these GPCRs have been reported to significantly alter anesthetic requirements in humans and animals (Segal et al., 1988; Seitz et al., 1990; Glass et al., 1997; Ishizawa et al., 2000a). Although this might include unrelated, parallel effects on the central nervous system, recent studies show that inhaled anesthetics can interfere with GPCR signaling in vitro (Durieux, 1995; Hennemann et al., 1998; Schotten et al., 1998), suggesting direct anesthetic effects.

Halothane, a clinically used volatile anesthetic, has two features that allow monitoring of binding. First, the photolabile carbon-bromine bond allows photolabeling (Eckenhoff and Johansson, 1997); second, the bromine atom can quench intrinsic protein fluorescence if it is near the fluorophore (Johansson et al., 1995). Both features allow determination of location of the anesthetic within the protein matrix. Because the abundance of rhodopsin in native retinal membrane preparation facilitates direct binding approaches, we used bovine rhodopsin as a structural homolog for other neuronal GPCRs to characterize the binding domain for this inhaled anesthetic. We reported previously that halothane binds to rhodopsin but not to its associated G protein (Ishizawa et al., 2000b). In this study, using a higher resolution approach, we provide evidence for halothane binding to the endogenous ligand binding site in rhodopsin.

Materials and Methods

Rod Disk Membranes Preparation. Fresh bovine retinas were dissected in room light. Rod disk membranes (RDM) were prepared by sucrose flotation in isotonic buffer (20 mM MOPS, 100 mM KCl, 6 mM MgCl₂, pH 7.0). Peripheral proteins were stripped by washing in hypotonic buffer (10 mM MOPS, 2 mM MgCl₂, 100 µM GTP, pH 7.0) (Panico et al., 1990). Estimated molar ratio of the protein in the RDM was 24:1 (rhodopsin/transducin) based on the relative mass in SDS-PAGE using reflective density. Steady-State Fluorescence and Absorption Spectra. For the fluorescence time-based measurements, the RDM samples were excited at 295 nm and 330 nm at 30-s intervals with continuous stirring. Data were recorded at 330 nm at 30-s intervals, as reported previously, to provide almost 100% of chromophore regeneration (Gibson et al., 1998). For digestion with Staphylococcus aureus glutamyl endopeptidase (V8 protease; ICN Biomedicals Inc., Aurora, OH), V8 protease was added to the final concentration of 1:1 (w/w) protease/rhodopsin. For proteolytic digestion and radiosequence analysis, the bleached and regenerated RDM samples were incubated with [¹⁴C]halothane at 0.75 mM in isotonic MOPS buffer. The samples were exposed to 254-nm light for 40 s. The labeled samples were washed with the buffer, and the pellets were then used for proteolytic digestion.

Steady-State Fluorescence and Absorption Spectra. All fluorescence measurements were performed with a spectrophotometer (RF-5301PC; Shimadzu Scientific Instruments, Inc. Columbia, MD) using a 10-mm-pathlength quartz cell at 25°C. For proteolytic fragments from enzymatic digestion was diluted in loading buffer. SDS-PAGE was performed in the modified Laemmli gels, and the gel was subsequently electroblotted to a polyvinylidene difluoride membrane (ProBlott Membranes; Applied Biosystems, Foster City, CA).

Automated N-terminal sequence analysis was performed on an Applied Biosystems model 473A protein sequencer (Foster City, CA) with an in-line 120A PTH analyzer. Blotted samples were directly loaded onto the chamber, and sequencing was performed using gas-phase trifluoroacetic acid to minimize possible hydrolysis. After conversion of the released amino acids to PTH-amino acids, the suspension was divided into two parts. One portion, approximately 30%, went to the PTH analyzer, whereas the remaining 70% was collected for scintillation counting. Yield of PTH amino acids was calculated from peak height compared with standards using the model 610A Data Analysis Program. Cysteine was not included in the standards. The analysis was done at least twice for each fragment.

Results

Halothane Binding to Opsin and Regenerated Isorhodopsin. Halothane binding to rhodopsin in RDM was initially studied using intrinsic protein fluorescence. Halothane decreased tryptophan fluorescence of the bleached RDM by 80% with a Kₐ value of 2.3 mM (95% CI, 1.8–2.8) (Fig. 1A). Tryptophan fluorescence of the RDM was also

wavelength for tryptophan, so inner filter corrections were not performed.

All UV/visible spectra were measured with a spectrophotometer (Cary300Bio; Varian Instruments, Walnut Creek, CA) using a 10-mm-pathlength 1.8-ml quartz cell at 25°C. RDM samples were equilibrated in sodium phosphate buffer at a rhodopsin concentration of 5.0 µM with increasing concentrations of halothane (0–4.0 mM). The time course of the increase in absorbance at 487 nm after addition of 9-cis-retinal (3-fold molar excess) was measured for 180 min.
that can be quenched, and K is intensity in the absence of halothane, Q is the total fluorescence intensity in the absence of halothane, \( K_D \) is the dissociation constant for the binding of anesthetic to rhodopsin. Values are means ± S.E.M (\( n = 5 \)). B, Stern-Volmer plots of halothane quenching. \( F_0 \) is the total fluorescence intensity that can be quenched, and \( K_{SV} \) is the dissociation constant for the binding of anesthetic to rhodopsin. Values are means ± S.E.M (\( n = 5 \)).

The reversibility of the effects of halothane on tryptophan fluorescence was examined to evaluate whether halothane-induced aggregation or precipitation of rhodopsin might be responsible for the observed tryptophan quenching. RDM samples in the presence of halothane was first measured fluorometrically, and then exposed to a stream of argon for 90 min in the dark to remove the anesthetic (Roberts and Dunker, 1993). These samples regained 105 ± 4% and 90 ± 10% of its tryptophan fluorescence for 2 mM and 12 mM halothane, respectively, compared with a control sample treated in the same manner (mean ± S.E.M., \( n = 3 \) for each). The tryptophan emission maximum after the treatment was not different from the value of the control.

To evaluate the environments surrounding the tryptophan residues in rhodopsin, experiments were performed with a water-soluble quencher, acrylamide. Acrylamide weakly quenched tryptophan fluorescence for bleached RDM and regenerated RDM (17% and 36%, respectively, at 0.8 M) in the absence of halothane (Fig. 2A). Acrylamide also caused the emission maximum to shift from 329.6 ± 0.2 nm to 326.8 ± 0.1 nm in the bleached RDM, and from 330.8 ± 0.2 nm to 331.1 ± 0.2 nm in the regenerated RDM. Halothane had no significant effect on this acrylamide-sensitive component (Fig. 2B), indicating that halothane does not bind near the more exposed tryptophan residue accessible to acrylamide. The recently reported crystal structure of bovine rhodopsin identifies Trp35 as the most solvent exposed tryptophan residue (Palczewski et al., 2000), and thus Trp35 is a reasonable candidate for quenching by acrylamide.

Similar quenching behavior suggests that halothane and retinal binding sites may overlap. To test for the predicted competition between halothane and the ligand retinal, we photolabeled RDM with \([^{14}C]\)halothane in the presence and absence of bound retinal. Incorporation of \([^{14}C]\)halothane into opsin (the receptor protein without bound retinal) was significantly inhibited by unlabeled halothane with an IC\(_{50}\) value of 1.1 mM (95% CI, 0.8–1.6) and a Hill coefficient of -1.4 (95% CI, -2.1 ~ -0.8) (Fig. 3). In regenerated isorhodopsin, although the IC\(_{50}\) was essentially unaltered (1.1 mM), label incorporation was significantly decreased. The calculated maximum stoichiometry was 1.08 (halothane/rhodopsin molar ratio) in opsin and 0.28 in isorhodopsin.
Halothane Binds to the Ligand Binding Site. The fluorescence and photoaffinity labeling data both suggest that halothane binds close to the ligand binding site in the transmembrane core of rhodopsin. However, hydrophobic volatile anesthetics have been shown to preferentially bind to the lipid-protein interface as well (Tang et al., 2000), which might alter fluorescence and ligand binding allosterically. Accordingly, we tested this possibility by performing proteolysis and radiosequence analysis of \(^{14}\text{C}\)halothane-photolabeled rhodopsin. A small fragment from enzymatic digestion with V8 protease, previously shown to include the site where photoreactive retinal analogs are incorporated (Zhang et al., 1994), was used (Fig. 4A, V8-S). Release of \(^{14}\text{C}\) was observed in cycle 26, indicating halothane photoincorporation at Trp265 (Fig. 4B). In the same fragment from regenerated isorhodopsin (retinal-bound), \(^{14}\text{C}\) release at Trp265 was significantly smaller than that released from opsin. Because the efficiency of Edman degradation is not 100%, the repetitive yield results in a well-known lag of the residue release (and therefore cpm), which grows in magnitude with the number of cycles. Therefore, most of the cpm release measured at cycle 27 and the cycles thereafter represents lag from cycle 26 and is unlikely to represent labeling of Leu266. Because we predicted that the acrylamide-sensitive, halothane-resistant component of the tryptophan fluorescence arose from Trp35, we subjected a proteolytic fragment containing this residue to radiosequencing as well (Fig. 4A, EndoLysC). In this EndoLysC fragment, there was no significant release of radioactivity in close vicinity to Trp35 in opsin or in isorhodopsin (Fig. 4C), confirming the importance of the more buried site, and eliminating the trivial explanation of photochemical selectivity being responsible for Trp265 labeling. Additionally, the present radiosequencing through helices 1 and 6 did not provide evidence that halothane preferentially binds to the residues at the lipid-protein interface.

The crystal structure of bovine rhodopsin confirms that Trp265 lines the core of the \(\alpha\)-helical bundle (Palczewski et al., 2000). Trp265 is known to interact with retinal (Lin and Sakmar, 1996; Kochendoerfer et al., 1997; Palczewski et al., 2000) and to play an important role in rhodopsin regeneration (Reeves et al., 1999). To illustrate the relationship between the retinal cavity formed in the interhelical space of rhodopsin and the photolabeled residues, the Gravitational Radiation Analysis and Simulation Package (GRASP; http://www.lsc-group.phys.uwm.edu/~ballen/grasp-distribution/) was used to render the cavity surface on the crystal structure of rhodopsin with retinal removed (Fig. 5) (Nicholls et al., 1991). This cavity is measured to be \(428\ \AA^3\), sufficient volume to accommodate at least two halothane molecules (volume of \(\sim 130\ \AA^3\)) but it is likely to at least partially collapse on removal of retinal. No other suitable cavity exists in the protein structure.

Fig. 3. Photoaffinity labeling of \(^{14}\text{C}\)halothane to bleached and regenerated RDM. Protection from \(^{14}\text{C}\)halothane labeling by unlabeled halothane is shown in opsin and isorhodopsin recovered from the gel of the bleached RDM and the regenerated RDM, respectively. Photoincorporation is presented as disintegrations per minute per nanomolar rhodopsin. The data were fitted to sigmoid dose-response curves (variable slopes) with nonlinear least-squares regression. Values are means \(\pm\) S.E.M. (\(n = 3\)). Insert shows rhodopsin band (Rh) in the stained gel electrophoresis of the bleached RDM (lane 1) and the regenerated RDM (lane 2). Lanes 3 and 4 are the resulting autoradiogram, which show dominant photoincorporation into the opsin band in the bleached RDM (lane 3) but significantly less incorporation into the isorhodopsin band in the regenerated RDM (lane 4).
Competition between Halothane and the Ligand. Finally, to confirm the predicted competitive interaction between halothane and the ligand retinal, retinal binding kinetics was monitored by the specific absorption wavelength for isorhodopsin after the addition of 9-cis-retinal. Halothane significantly prolonged isorhodopsin formation in a concentration-dependent manner, indicating that halothane inhibits retinal binding to opsin (Fig. 6; Table 1). The kinetics of the fluorescence decrease produced by retinal binding was also prolonged in the presence of halothane (Table 1). These data not only confirm competition between halothane and retinal at the retinal binding site in rhodopsin but also predict specific in vivo functional changes (see Discussion). Although we noted that the increase in the absorbance was slower than the fluorescence decrease, the spectroscopic probes report different endpoints of the regeneration process of isorhodopsin, and the data suggest that quenching of tryptophan fluorescence is established at an earlier stage of chromophore regeneration.

Discussion

Using several different approaches, we have shown here that a general inhaled anesthetic binds to the endogenous ligand binding site and competitively inhibits the ligand binding in a prototypical GPCR. Photoincorporation of
[14C]halothane at Trp265 indicates unambiguous location of halothane in the interhelical core in opsin, which is consistent with the photoaffinity stoichiometry of 1 for halothane in opsin. The crystal structure of bovine rhodopsin has confirmed that the ligand-binding cavity can accommodate halothane in the core formed by the α-helical bundle.

Competition between halothane and the ligand retinal predicts that the anesthetic may interfere with retinal binding in a functionally apparent manner. Indeed, the functional importance of this binding interaction is shown by a recent in vivo finding that rhodopsin regeneration in mice and rats was significantly inhibited under halothane anesthesia at 1.5–1.8% (v/v), corresponding to about 0.4 mM at 37°C (Keller et al., 2001). Dark adaptation of vision, in which retinal chromophore regeneration plays an important role, was also reported to be retarded in humans and monkeys under halothane anesthesia (van Norren and Padmos, 1975). Our data are consistent with other studies showing functional effects of volatile anesthetics on GPCRs in vitro as well. For example, halothane competitively inhibited muscarinic and thromboxane A2 signaling monitored with Ca2+-activated Cl− currents in Xenopus laevis oocytes, but it had no effect on intracellular signaling pathways, indicating that halothane interacts with the receptor and/or receptor-G protein coupling (Durieux, 1995; Honemann et al., 1998). Halothane also decreased ligand binding affinity for the β-adrenergic receptor in rat myocardium, and reduced positive inotropic potency of its agonist (Schotten et al., 1998). Although the effect of inhaled anesthetics on protein kinase C (PKC) or on PKC phosphorylation sites of the receptor was suggested in the metabotropic glutamate receptor, direct interactions of anesthetics with the hydrophobic domain of the receptor cannot be eliminated because of the lack of the concentration-effect relationships between PKC inhibitors/activators and anesthetics (Minami et al., 1998). Further studies may need to define anesthetic actions at the downstream of a variety of GPCR signaling cascades.

It is interesting that the effective concentrations of halothane in these previous in vivo and in vitro studies seem to be lower than those describing the binding relationship in this study (1.1–2.3 mM, equivalent to the gaseous concentration of 4 to 9%). Dark adaptation was significantly altered at the lowest halothane concentration of 0.2% (van Norren and Padmos, 1975). Halothane inhibited muscarinic signaling in oocytes with an IC50 of 0.3 mM (Durieux, 1995). These differences probably reflect the enormous signal amplification and the well-known displacement of concentration-effect curves from receptor-occupancy profiles in the GPCR systems (Ross, 1996). In this context, it is particularly interesting that halothane almost completely inhibited retinal regeneration in mice at 1.8% (Keller et al., 2001), the same phenomenon that we observed using membrane preparations at higher halothane concentrations. The explanation for this difference in halothane sensitivity is not clear, but could be caused by species differences or by altered functioning of this receptor in the in vitro membrane preparation.

The site of halothane binding identified in this study may represent a common anesthetic binding motif in many GPCRs. First, the residue identified as most contributing to the site for halothane, Trp265, is one of the most conserved residues in more than 199 unique sequences of the GPCRs in the rhodopsin family (Baldwin et al., 1997). Second, a similar interhelical domain is created with a sufficient number of conserved residues in the transmembrane portion in other GPCRs (Baldwin et al., 1997). It is interesting that most of these highly conserved residues face the interior of the molecule in the crystal structure of rhodopsin (Palczewski et al., 2000), confirming a similar molecular environment in the receptor.

**Fig. 6.** Effects of halothane on retinal binding kinetics. Retinal binding to opsin in the RDM was monitored by the increase in the specific absorption wavelength for isorhodopsin after addition of 9-cis-retinal in the dark. Halothane did not affect the baseline spectrum (700–350 nm) for the bleached RDM, and this baseline spectrum was subtracted from subsequent spectra after addition of retinal. The 3-fold molar excess of 9-cis-retinal itself has an absorption peak at 380 nm and its absorbance was 0.013 at 487 nm. The curves through the data points were generated using a two-phase exponential association.

**TABLE 1**

Effects of halothane on retinal binding kinetics

<table>
<thead>
<tr>
<th>Halothane</th>
<th>0.0 mM</th>
<th>1.0 mM</th>
<th>2.0 mM</th>
<th>4.0 mM</th>
<th>6.0 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance Increase Half-Time</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (s)</td>
<td>47 ± 6</td>
<td>53 ± 2</td>
<td>63 ± 5</td>
<td>76 ± 7†</td>
<td></td>
</tr>
<tr>
<td>T2 (min)</td>
<td>17 ± 2</td>
<td>23 ± 4</td>
<td>25 ± 2</td>
<td>35 ± 6‡</td>
<td></td>
</tr>
<tr>
<td>Fast component (%)</td>
<td>68 ± 3</td>
<td>57 ± 7</td>
<td>50 ± 3*</td>
<td>38 ± 2†</td>
<td></td>
</tr>
<tr>
<td>Fluorescence Decrease Half-Time</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (s)</td>
<td>8.0 ± 0.6</td>
<td>9.3 ± 0.5</td>
<td>12.4 ± 1.2‡</td>
<td>13.6 ± 1.0‡</td>
<td></td>
</tr>
<tr>
<td>T2 (min)</td>
<td>3.4 ± 0.3</td>
<td>5.3 ± 0.5</td>
<td>6.8 ± 0.9*</td>
<td>12.2 ± 1.1‡</td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.05 compared with the absence of halothane [one-way analysis of variance (ANOVA) followed by Student-Neuman-Keuls test].
†P < 0.05 compared with halothane 1.0 mM (one-way ANOVA followed by Student-Neuman-Keuls test).
‡P < 0.05 compared with halothane 2.0 mM (one-way ANOVA followed by Student-Neuman-Keuls test).
core in these GPCRs. Furthermore, with the use of designed four-α-helical bundles (Johansson et al., 1998, 2000), and other soluble proteins with hydrophobic cavities (Eckenhoff et al., 2000; Eriksson et al., 1992), it has been shown that preformed accessible cavities are important features to facilitate anesthetic binding. Although inhaled anesthetics have divergent chemical structures, the molecules are relatively small (90–160 Å³) and have few interactive atoms or groups to provide much selectivity, compared with other molecules that interact with GPCRs, such as biogenic amines and retinal. All these data support the notion that inhaled anesthetic binding in the interhelical core is a feature of general significance in the GPCR family.

A common binding motif in this class of receptor may also suggest a common mechanism of action of inhaled anesthetics. Although several distinct modes of ligand-receptor interactions are reported for GPCRs, small ligands, such as biogenic amines, eicosanoids, enter and bind in the transmembrane core of their GPCRs as retinal does (Strader et al., 1994; Ji et al., 1998). Covalent attachment of the ligand is indeed unique to rhodopsin, but the binding site for catecholamine in the β-adrenergic receptor is thought to be remarkably similar to the binding site for retinal in rhodopsin (Strader et al., 1994; Sakmar, 1998). Cholinergic ligands have also been reported to interact with aromatic as well as polar residues, such as tyrosine, in the core of the muscarinic receptor (Hulme et al., 1999). Our results thus suggest that disruption of signal transduction through competition with endogenic ligands in the core may be a common mechanism of the action of an inhaled anesthetic like halothane at these GPCRs. Moreover, it has been reported that the ligands that bind to the transmembrane core of the metabotropic glutamate receptor, another family of the GPCRs, allosterically inhibit receptor signaling after agonist binding to the extracellular domain (Litschig et al., 1999; Pagano et al., 2000). This suggests that anesthetic occupancy of this core region may have both competitive and allosteric effects on receptor function. Key features of general anesthesia, such as loss of consciousness and anticonvulsion, as well as anesthetic “side” effects, which include a multitude of cardiovascular and autonomic features, could be explained by a wide spectrum of the roles of GPCR signaling in the central and peripheral nervous systems.

It is important to note that there are striking differences in the effective concentrations between inhaled anesthetics and other pharmacological agents. For example, in contrast to the aqueous EC50 of inhaled anesthetics, ranging between 0.2 and 1.0 mM, opioids and benzodiazepines are effective in nanomolar concentrations. This has led to the interpretation that the inhaled anesthetics may interact with multiple protein sites to produce anesthetic state. Our present data also suggest possible interactions of inhaled anesthetics with other neuronal membrane proteins as well as GPCRs. Because α-helical bundles are a commonly found motif in many membrane proteins, occupancy of interhelical cavities could cause changes in helical arrangement, disruption of oligomerization equilibrium, or alteration in dynamic behavior, all of which may further explain divergent physiological effects of these widely used drugs.

Acknowledgments

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