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Pharmacology

An inhalational anesthetic binding domain in the nicotinic acetylcholine receptor

(halothane/isoflurane/photoaffinity labeling/*Torpedo*)

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ABSTRACT To determine inhalational anesthetic binding domains on a ligand-gated ion channel, I used halothane direct photoaffinity labeling of the nicotinic acetylcholine receptor (nAChR) in native *Torpedo* membranes. [^{14}C]Halothane photoaffinity labeling of both the native *Torpedo* membranes and the isolated nAChR was saturable, with K_d values within the clinically relevant range. All phospholipids were labeled, with greater than 95% of the label in the acyl chain region. Electrophoresis of labeled nAChR demonstrated no significant subunit selectivity for halothane incorporation. Within the α-subunit, greater than 90% of label was found in the endoprotease Glu-C digestion fragments which contain the four transmembrane regions, and the pattern was different from that reported for photoactivatable phospholipid binding to the nAChR. Unlabeled halothane reduced labeling more than did isoflurane, suggesting differences in the binding domains for inhalational anesthetics in the nAChR. These data suggest multiple similar binding domains for halothane in the transmembrane region of the nAChR.

Electrophysiologic studies have suggested that the superfamily of ligand-gated ion channels is important in mediating the action of inhalational anesthetics and other depressant drugs (1–3), but it remains unclear whether the anesthetic action is mediated through interactions with the lipid or protein component of the neuronal membranes that contain these channels. If these anesthetics act directly on protein, as suggested by recent studies (1, 4, 5), no evidence has emerged to implicate a specific binding domain, or even a class of domain. Understanding the mechanism of inhalational anesthetic action would be assisted by precise identification of their sites of action. However, the low affinity, volatility, and rapid binding kinetics of these agents have frustrated efforts to define their binding sites. In an attempt to identify anesthetic binding domains, I have used a recently described and validated direct photoaffinity labeling approach for halothane (6, 7), a clinically important inhalational anesthetic, to investigate the distribution of label in native *Torpedo* membranes, with specific attention to the nicotinic acetylcholine receptor (nAChR) as a representative of the ligand-gated ion channel family.

MATERIALS AND METHODS

Binding Isotherms. Electroplax membranes from *Torpedo nobiliana* were prepared as described previously (8, 9) and incubated with increasing concentrations of [^{14}C]halothane (specific activity 6.6 mCi/mmol; DuPont/NEN; 1 mCi = 37 MBq) with or without excess unlabeled halothane in quartz cuvettes with continuous stirring and were photoaffinity labeled for 20 sec as previously described (6, 7). Bound label was separated from free by using vacuum filtration and determined with liquid scintillation counting, from which binding isotherms were constructed. Similar isotherms were also constructed for purified nAChR (in the absence of lipid, see below), which required precipitation with trichloroacetic acid after photolysis and prior to filtration.

Lipid Analysis. In some aliquots of membranes labeled with 0.2 mM [^{14}C]halothane, lipid and protein labeling was separated by extraction of membrane aliquots (10) into organic and aqueous fractions, respectively. Nonspecific labeling of each component was determined by including 6 mM halothane in the photolysis system. The ability of another inhalational anesthetic, isoflurane (7.0 mM), to compete with halothane binding was also investigated. The organic phase was spotted on silica gel-G plates, and thin-layer chromatograms were developed with $CHCl_3$/CH_3OH/29% (wt/wt) NH_4OH/H_2O, 65:35:2.5:2.5 (vol/vol). Lipids were eluted from scraped spots with $CHCl_3$/CH_3OH 1:1 and assayed for phosphorus, cpm (scintillation counting), and cholesterol by an enzymatic assay (Sigma kit 352). After elution and determination of specific labeling, each phospholipid class was subjected to a mild alkaline hydrolysis and extraction (11) to determine distribution of label between head group and acyl chain region.

Receptor Binding. Larger samples of *Torpedo* membranes photolabeled with a higher specific activity [^{14}C]halothane (51.4 mCi/mmol, 0.2 mM) in the presence or absence of 100 μM carbamoylcholine, 5 mM halothane, or 7 mM isoflurane were solubilized, and the nAChR was extracted and purified with *Naja*-toxin-linked Sepharose gel as previously reported (8). SDS/PAGE of the labeled and purified receptors was followed by gel autoradiography and quantitation of nAChR subunit label incorporation by cutting out subunit bands, dissolving them in 30% (wt/wt) H_2O_2, and scintillation counting. To further characterize the binding location of [^{14}C]halothane within the nAChR, limited proteolysis of the isolated α-subunit with endoprotease Glu-C was used (12). Briefly, the 40-kDa band (α-subunit) was excised from SDS/8% PAGE minigels and placed in the wells of a large 16% polyacrylamide sequencing gel. Five micrograms of endoprotease Glu-C (*Staphylococcus aureus* V8) was added to the well, and the electrophoresis was initiated at constant voltage (70 mV). When the protein was half-way through the stacking gel, electrophoresis was interrupted for 30 min to allow proteolysis and was then restarted at constant current (24 mA). There was insufficient labeling for autoradiography, so the gel bands were excised and dissolved in 30% H_2O_2, and label incorporation was determined by scintillation counting.

RESULTS AND DISCUSSION

Binding Isotherms. Binding isotherms showed clear evidence of saturable binding in both the native membranes and the purified receptor (Fig. 1), the principle difference being the relatively higher nonspecific component in the membranes,

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Abbreviation: nAChR, nicotinic acetylcholine receptor.

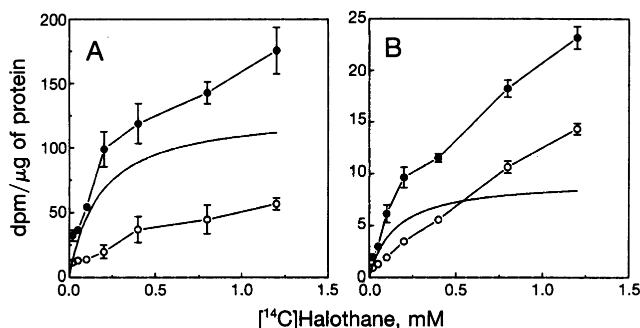

FIG. 1. Binding isotherms for [^{14}C]halothane to isolated receptors (*A*) and *Torpedo* P2 membrane fraction (*B*). In both graphs the filled symbols are total binding and the open symbols are nonspecific binding (in the presence of 6 mM unlabeled halothane). The curve is a nonlinear least-squares regression of the specific binding data (total − nonspecific) to a rectangular hyperbola; apparent K_d values are 0.18 ± 0.04 mM for *A* and 0.15 ± 0.04 mM for *B*.

presumably due to the presence of lipid. Apparent dissociation constants are in the high micromolar range, consistent with the functional effects of these agents in this receptor system and *in vivo* (13).

Lipid Analysis. Lipid/protein separation of the native membranes demonstrated 45% of label in the aqueous (protein) fraction, with the remainder in the organic phase. Unlabeled halothane (5 mM) reduced aqueous label incorporation by 79%, and organic label by 68%. Isoflurane, however, reduced aqueous label by only 40% and did not appreciably reduce the organic label. Thin-layer chromatograms showed that all lipids were labeled at a stoichiometry of between 1:130 and 1:60 (halothane to phospholipid; Table 1), and 94% ± 4% of label was localized to the acyl chain region of all phospholipid species. This predominance of hydrophobic core labeling renders unlikely the hypothesis of extensive halothane binding to the water/phospholipid interface (14).

Receptor Binding. Electrophoresis of nAChR labeled with the higher specific activity halothane demonstrated an overall incorporation of about 2.5 pmol of halothane per pmol of nAChR (assuming the mass of nAChR is 290 kDa) and further showed that all subunits were labeled (Fig. 2), with a slight preference of the α and β over the γ and δ subunits (Table 2), assuming the known stoichiometry for this receptor of $\alpha_2\beta\gamma\delta$. This may, however, reflect the relatively lower stability of the γ and δ subunits after isolation as compared with the α and β. The agonist carbamoylcholine did not significantly alter the pattern or degree of subunit labeling, suggesting that the conformational changes associated with receptor function and desensitization do not alter the binding domain for halothane and that the agonist site is not a likely candidate for halothane binding. Similarly, the high-affinity antagonist α-bungarotoxin

Table 1. Label incorporation into *Torpedo* membranes at 0.2 mM [^{14}C]halothane

Lipid	[^{14}C]Halothane incorp., pmol/nmol*	Nonspecific incorp., %†
Phosphatidylcholine	7.5 ± 0.8	30
Phosphatidylethanolamine	16.6 ± 2.1	26
Phosphatidylserine	16.4 ± 5.0	19
Cholesterol	1.8 ± 0.3	42

Statistics: Phosphatidylethanolamine or phosphatidylserine label > phosphatidylcholine or cholesterol label ($P < 0.01$; ANOVA and Scheffe's test).
*Label incorporation expressed as mean (±SEM) pmol of halothane per nmol of lipid for both the phospholipids and cholesterol for at least three separate experiments.
†Label incorporation in the presence of 5.0 mM halothane expressed as percent of total.

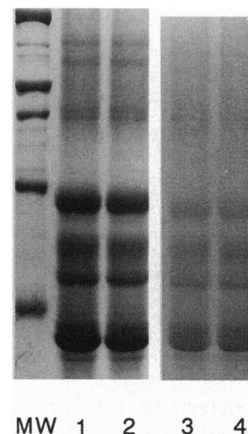

FIG. 2. SDS/10% PAGE of the purified nAChR labeled with (lanes 1 and 3) and without (lanes 2 and 4) 100 μM carbamoylcholine. Lanes 1 and 2 are stained with Coomassie blue and lanes 3 and 4 are the autoradiogram of the same gel. Lane MW size markers, from the top, are myosin (200.0 kDa), β-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), and ovalbumin (45.0 kDa) (Bio-Rad). The prominent bands from the bottom are α, β, γ, and δ subunits of the nAChR. Material at the top of the gel is presumably cross-linked receptor subunits. UV exposure to complete photolysis of halothane was deliberately not carried out because long exposures at this short wavelength cause cross-linking of proteins and subsequent difficulty with separation.

(BGT) did not alter [^{14}C]halothane labeling, and [^{3}H]BGT binding itself was not altered by prior halothane photoaffinity labeling of *Torpedo* membranes (data not shown). Although excess unlabeled halothane reduced [^{14}C]halothane binding to the nAChR by 75% and isoflurane reduced it by about 50%, no significant alteration in subunit distribution was noted. This suggests that the binding sites for the different inhalational anesthetics are similar but not identical.

The endoprotease Glu-C digestion permitted the determination of halothane incorporation in broad domains of the α-subunit. The digestion fragments were essentially identical to those found by others using this technique (15, 16) and consisted of four major groups of bands on SDS/PAGE (Fig. 3). Approximately 90% of the label in fragments of less than 20 kDa (a more complete digestion) was found in the 18- to 18.8-kDa and the 7.6- to 11.5-kDa regions of a 16% polyacrylamide gel (Table 3). Other major digestion fragments at 15–16 kDa and 4.8–5.5 kDa had incorporated little or no label. Carbamoylcholine did not alter this pattern, but halothane and isoflurane appeared to reduce labeling of the 7.6- to 11.5-kDa region more than the other fragments (Fig. 4 and Table 3). The ≈19-kDa band (previously called α-V8-20) has been shown to start at Ser-162/Ser-173, and it is long enough to contain the MI, MII, and MIII putative transmembrane sequences of the α-subunit (15). Similarly, the 10- to 12-kDa fragments (α-V8-10) are reported to contain the MIV transmembrane sequence (15, 16). The poorly labeled but prominent 15- to 16-kDa band represents the N terminus and contains most of the extracellular domain of the receptor subunit, and the small (4- to

Table 2. Subunit labeling by [^{14}C]halothane

Conditions	Total [^{14}C]halothane incorp., pmol/pmol of receptor	Relative subunit incorp. α:β:γ:δ
Control	2.5 ± 0.4	1:1:0.9:0.8
Halothane (6 mM)	0.6 ± 0.2	1:1:0.8:0.7
Isoflurane (7 mM)	1.3 ± 0.2	1:1.1:0.8:0.8

Statistics: Total incorporation, control > isoflurane > halothane ($P < 0.05$; ANOVA and Scheffe's test) and α and β incorporation > γ and δ ($P < 0.05$) (*t* test). Results are mean ± SEM.

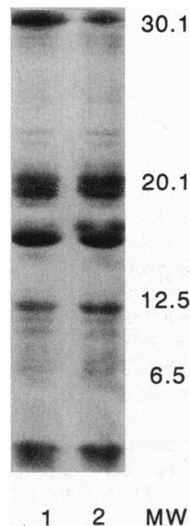

FIG. 3. Limited proteolysis of the nAChR α-subunit. A Coomassie-blue-stained gel of fragments labeled with halothane in the presence of 100 μM carbamoylcholine (lane 1) or in the absence of carbamoylcholine (lane 2) is shown. MW indicates marker (kDa) locations.

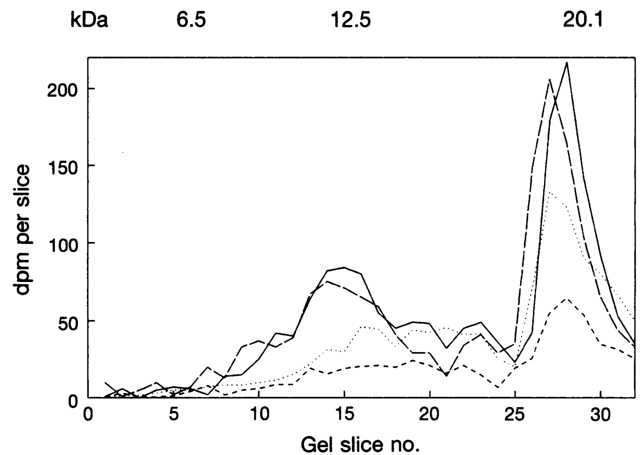

FIG. 4. Distribution of label ([^{14}C]halothane) in endoprotease Glu-C fragments of the α-subunit photoaffinity labeled with no addition (—), in the presence of 100 μM carbamoylcholine (— —), in the presence of 6 mM halothane (- - -), or in the presence of 7 mM isoflurane (· · ·). Labeling and digestion were conducted as described for Fig. 3. The gel was cut into 2-mm slices, which were dissolved with 30% H_2O_2, and radioactivities were measured by liquid scintillation counting.

6-kDa) fragments presumably represent the cytoplasmic domain and smaller pieces of the extracellular domain. The simplest explanation of this labeling pattern is that the majority of halothane is binding to the putative transmembrane sequences of the α-subunit. It also demonstrates that multiple binding sites for halothane exist in the nAChR. The relatively more conserved nature of the transmembrane sequences between the nAChR subunits, especially MI–III, combined with the quantitatively similar subunit labeling by halothane (Fig. 1), is also consistent with transmembrane domain localization of halothane. It is not clear, however, if *all* the transmembrane segments are labeled. Because the MII region is thought to line the ion channel of this receptor complex (17, 18) and is therefore less hydrophobic than the others, it seems less likely that the MII region is a binding domain for halothane. The lack of functional competition of inhalational anesthetics with channel-blocking drugs (13) supports this speculation that halothane does not bind in the MII region, but verification will require further digestion of the α-V8-20 fragment.

Chemical incorporation of the halothane photolysis product into the putative transmembrane sequences of the receptor is a probable consequence of equilibrium binding of halothane at the lipid/protein interfaces. Also in agreement with interfacial binding is a recent study which showed that the lipid affinity of the nAChR is decreased in the presence of anesthetics (19), and other work with simpler lipid/peptide models that suggested anesthetic localization at the lipid/protein boundary (20, 21). Finally, an interfacial binding site for anesthetics on the membrane protein bacteriorhodopsin has been suggested from x-ray diffraction studies (22). Taken together, the simplest interpretation of these data is that halothane binds to the transmembrane sequences through localization at the lipid/protein interface of the nAChR subunits. However, if these interfaces were the only site of halothane binding, a similar labeling distribution of the nAChR should occur with photoactivatable phospholipids (16, 23). But when the nAChR is labeled with a photoactivatable phosphatidylserine analogue, MIV is labeled to a far greater extent than MI, MII, and MIII. Smaller ligands like halothane may penetrate between or into the other transmembrane sequences, or between adjacent subunits to produce significant labeling of the MI–III-containing fragment. Another small photoactivatable hydrophobic probe, 3-trifluoromethyl-3-(*m*-[^{125}I]iodophenyl)diazirine (TID), was also found to preferentially label the fragment containing MI, MII, and MIII (15), similar to our results with halothane, but because TID binding was carbamoylcholine sensitive and selective for the γ-subunit, halothane sites must be distinct from those of TID.

Table 3. Label incorporation into V8 proteolysis fragments of nAChR α-subunit

Fragment, kDa	% of total label*		
	Control	Halothane	Isoflurane
18.0–18.8	54 ± 1	54 ± 3	48 ± 4
14.9–15.7	9 ± 3	16 ± 1	16 ± 3
7.6–11.4	35 ± 1	25 ± 2	33 ± 5
4.8–5.5	2 ± 2	5 ± 1	3 ± 1

*Determined as a mean (±SEM) percent of total radioactivity in fragments of less than 20 kDa; the sum is 100% in all cases. Control, [^{14}C]halothane only; Halothane, 6 mM unlabeled halothane added; Isoflurane, 7 mM isoflurane added.

It is not yet clear if the action of halothane (or other inhalational anesthetics) on this receptor (24) is due to binding to one, all, or any of the sites around the transmembrane sequences. However, it is clear that these putative transmembrane sequences, especially MI–III, are intimately involved in the gating, conductance, and selectivity of this ion channel (25–27). These domains are therefore attractive candidates for the functional sites of halothane and possibly other inhalational anesthetics. The inability of isoflurane to fully compete with halothane labeling is surprising for two reasons: first, the functional effects of these two inhalational anesthetics on this receptor are similar at similar concentrations, and second, isoflurane has been shown to compete with halothane binding in other proteins when photoaffinity as well as more conventional NMR approaches were used (28). This suggests that inhalational anesthetic binding sites, while similar in character, may be sufficiently different to provide a basis for the well-known qualitative differences in action. This specificity also implies action through direct protein interactions, consistent with our finding of a larger specific binding component on the nAChR as compared with the lipid. Similar inhalational anesthetic binding domains may exist in other central nervous system ion channels in the same superfamily, such as the γ-aminobutyric acid- or glycine-gated chloride channels, be-

cause within this family, the transmembrane segments are more highly conserved than the extramembranous segments.

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1. Franks, N. P. & Lieb, W. R. (1994) *Nature (London)* **367,** 607–614.
2. Tannelian, D. L., Kosek, P., Mody, I. & MacIver, B. (1993) *Anesthesiology* **78,** 757–776.
3. Jones, M., Brook, P. & Harrison, N. (1992) *J. Physiol.* **449,** 279–293.
4. Richards, C. D., Martin, K., Gregory, S., Keightley, C. A., Hesketh, T. R., Smith, G. A., Warren, G. B. & Metcalfe, J. C. (1978) *Nature (London)* **276,** 775–779.
5. Franks, N. P. & Lieb, W. R. (1991) *Science* **254,** 427–430.
6. El-Maghrabi, E. A., Eckenhoff, R. G. & Shuman, H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 4329–4332.
7. Eckenhoff, R. G. & Shuman, H. (1993) *Anesthesiology* **79,** 96–106.
8. Eldefrawi, M. & Eldefrawi, A. (1973) *Arch. Biochem. Biophys.* **159,** 362–373.
9. Chak, A. & Karlin, A. (1992) *Methods Enzymol.* **207,** 546–555.
10. Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917.
11. Dawson, R. M. C. (1960) *Biochem. J.* **75,** 45–53.
12. Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. K. (1977) *J. Biol. Chem.* **252,** 1102–1106.
13. Dilger, J. P. & Vidal, A. M. (1994) *Mol. Pharmacol.* **45,** 169–175.
14. Ueda, I. (1991) in *Advances in Membrane Fluidity* eds. Aloia, R., Curtain, C. & Gordon, L. (Wiley-Liss, New York), Vol. 5, pp. 15–33.
15. White, B. H. & Cohen, J. B. (1988) *Biochemistry* **27,** 8741–8751.
16. Blanton, M. P. & Wang, H. H. (1990) *Biochemistry* **29,** 1186–1194.
17. Hucho, F. & Hilgenfeld, R. (1989) *FEBS Lett.* **257,** 17–19.
18. Leonard, R. J., Labarca, C. G., Charnet, P., Davidson, N. & Lester, H. A. (1988) *Science* **242,** 1578–1581.
19. Frazer, D., Louro, S. R. W., Horvath, L. I., Miller, K. W. & Watts, A. (1990) *Biochemistry* **29,** 2664–2669.
20. Nakagawa, T., Hamanaka, T., Nishimura, S., Uruga, T. & Kito, Y. (1994) *J. Mol. Biol.* **238,** 297–301.
21. Jorgensen, K., Ipsen, J. H., Mouritsen, O. G. & Zuckermann, M. J. (1993) *Chem. Phys. Lipids* **65,** 205–216.
22. Veiro, J. A. & Hunt, G. R. A. (1985) *Chem. Biol. Interact.* **54,** 337–348.
23. Giraudat, J., Montecucco, C., Bisson, R. & Changeux, J. P. (1985) *Biochemistry* **24,** 3121–3127.
24. Dilger, J. P., Vidal, A. M., Mody, H. I. & Liu, Y. (1994) *Anesthesiology* **81,** 431–442.
25. Tobimatsu, T., Fujita, Y., Fukuda, K., Tanaka, K.-I., Mori, Y., Konno, T., Mishina, M. & Numa, S. (1987) *FEBS Lett.* **222,** 56–62.
26. Lo, D., Pinkham, J. & Stevens, C. (1991) *Neuron* **6,** 31–49.
27. Li, L., Schuchard, M., Palma, A., Pradier, L. & McNamee, M. (1990) *Biochemistry* **29,** 5428–5436.
28. Dubois, B. W., Cherian, S. F. & Evers, A. S. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 6478–6482.