

EFFECTS OF HALOTHANE ON THE NICOTINIC
ACETYLCHOLINE RECEPTOR FROM *TORPEDO*
CALIFORNICA

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Abstract—To determine whether the binding of anesthetics to key membrane receptors is a plausible mode of action, we modeled the effect of the general anesthetic halothane in the nicotinic acetylcholine receptor membrane system isolated from *Torpedo californica*. Our results demonstrated that halothane inhibits the binding of [³H]phencyclidine ([³H]PCP) to the acetylcholine receptor. The inhibition was reversible, concentration dependent, and had an equilibrium dissociation constant (K_d) of 2.2% atm halothane at 25°. Double-reciprocal plots of the halothane effects at various phencyclidine (PCP) concentrations imply that, under equilibrium conditions, halothane inhibits [³H]PCP binding competitively. In contrast, results from kinetic studies showed that the rate of PCP dissociation is highly sensitive to halothane with $EC_{50} = 0.8\%$ atm halothane in nitrogen. Several possible interpretations are discussed; however, the basic observation was that the kinetics of [³H]PCP binding to the nicotinic acetylcholine receptor was affected by halothane at low concentrations in this model system.

Key words: general anesthetic; halothane; nicotinic acetylcholine receptor; phencyclidine; PCP; *Torpedo californica*

Although electrophysiological studies show that general anesthetics affect the function of synaptic proteins [1–5], the mechanisms by which these effects are mediated remain unknown. Recent studies of general anesthetics have not resolved the controversy on whether anesthetics affect membrane lipids or bind directly to membrane proteins [6–8] to produce anesthesia. The correlation between lipid partition coefficients of anesthetics and their potencies suggests that the lipid domain may be the primary site of anesthetic action. However, phase changes in the lipid bilayer at clinical concentrations of anesthetics are too small to account for the phenomenon of anesthesia [9]. Direct interaction of the anesthetic with key proteins has been proposed; the recent report on the stereoselectivity of isoflurane is consistent with the protein hypothesis [10].

Halothane interacts with the soluble protein luciferase and competes with substrate binding of the enzyme [11], but no evidence exists for a direct binding of halothane to integral membrane proteins. Inhaled anesthetics as well as local anesthetics and PCP§ induce desensitization in the nicotinic acetylcholine receptor [12–16], although a relatively high concentration of halothane (3% atm) is required to induce receptor desensitization as measured by ligand binding at the agonist site [15]. The high-affinity PCP binding site is coupled allosterically to the agonist sites [17] and is located 21–35 Å from the agonist sites [18]. [³H]PCP binds at high affinity

(0.4 μM) to the receptor in the presence of agonists and has an approximate stoichiometry of one PCP per receptor-channel [12, 19]. We show here that clinical concentrations of halothane inhibited the binding of [³H]PCP to the acetylcholine receptor, thus providing a possible model of anesthetic-receptor interaction.

MATERIALS AND METHODS

Enriched preparations of nicotinic acetylcholine receptors were isolated from *Torpedo californica* as previously described [12, 20].

In our experiments, halothane was metered by an OHMEDA anesthetic vaporizer, calibrated by mass spectrometry and monitored by gas chromatography during the experiment. Halothane in nitrogen (10 L/min) flowed over a thin layer of membrane suspension (the thin layer was created by rotating 20 mL of the membrane suspension in a 100-ml round-bottom flask).

The radioligand assays used [³H]PCP to monitor binding of PCP to the high-affinity PCP site in the nicotinic acetylcholine receptor. The control level (or maximum binding) was defined as the total [³H]-PCP binding minus the non-specific binding of [³H]-PCP. Non-specific binding was obtained in an identical incubation mixture, but with the addition of unlabeled PCP at a concentration 1000-fold greater than [³H]PCP. The fraction bound was the specific binding of [³H]PCP at each experimental point divided by maximum binding. In binding assays, [³H]PCP was incubated with the membrane suspension for 30 min before halothane was applied. The incubation mixture (1 mL) contained 0.3 mg

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§ Abbreviations: PCP, phencyclidine; MOPS, 4-morpholinepropane sulfonic acid; and [³H]PCP, [³H]phencyclidine.

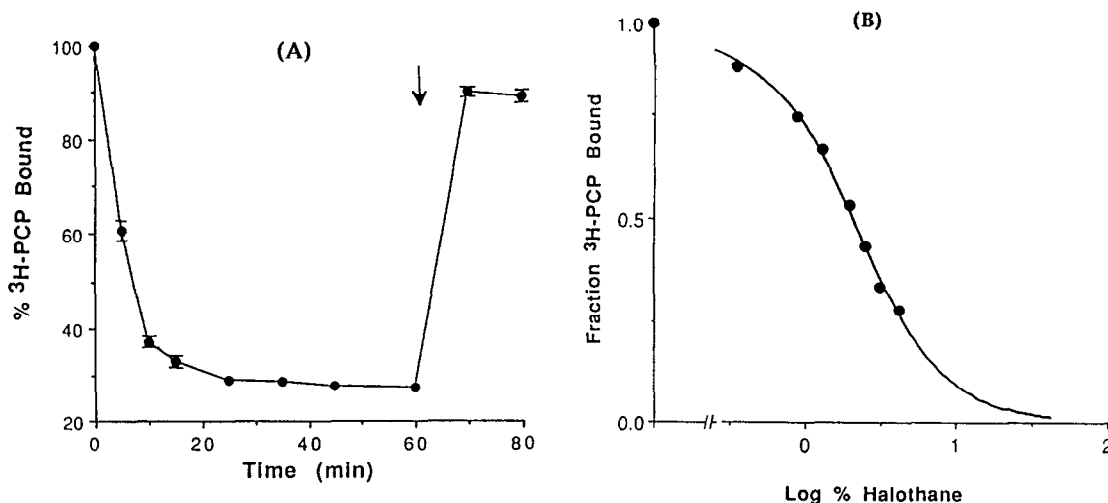


Fig. 1. Inhibition of [^3H]PCP binding to the nicotinic acetylcholine receptors by halothane under equilibrium binding conditions. (A) Effect of 2.5% (atm) of halothane in nitrogen on [^3H]PCP binding to receptors at 25° . The error bars represent the SD of 9 samples at each point (the error bars were deleted where they were shorter than the closed circle). The abscissa represents time after administration of halothane. During the recovery phase (indicated by arrow), the incubation mixture was exposed only to a stream of nitrogen gas at the same flow rate. (B) Inhibition of [^3H]PCP binding at each halothane concentration (incubated for 20 min).

protein (approximately 0.5 nmol receptor/mg protein), 10 nM [^3H]PCP, 1 mM carbamylcholine, 100 mM NaCl, 0.1 mM EDTA, 0.02% NaN_3 , and 10 mM HOPS buffer, pH 7.4. An identical incubation mixture was used to determine non-specific binding, but in the presence of 10 μM unlabeled PCP. The non-specific binding was typically 7% of the maximum (with non-specific scintillation counts at about 2500 cpm). In this experiment, aliquots of incubation mixture were removed and filtered quickly (< 1 sec) through a glass fiber filter (Whatman GF/B), and the filter was washed twice with 2 mL of cold buffer solution. The filter was then placed in scintillation fluid (Biosafe II), and radioactivity was counted the next day. In these experiments, a relatively high carbamylcholine concentration (1 mM) was used to ensure that the receptors exhibited uniformly high affinity for [^3H]PCP.

The dissociation rate constant, k , was determined by adding an equal volume of a pre-equilibrated halothane solution, which also contained a 200- to 2000-fold concentration of non-radioactive PCP, to a mixture of receptors equilibrated with 10 nM [^3H]PCP. Experiments were also carried out where receptors were pre-equilibrated with halothane before non-radioactive PCP was added. Pre-incubating the receptors with halothane or adding the halothane with PCP did not produce substantial differences in the value of the dissociation rate constant measured (the eventual halothane effects were 1 and 0.8% halothane, respectively). Aliquots of the freshly mixed solution were filtered and washed (< 1 sec) with cold buffer before counting the radioactivity. The exponential decay of bound [^3H]PCP in the receptor was fitted with the equation, $y = A_1 e^{-k_1 t} + A_2 e^{-k_2 t}$, where y is the percent [^3H]-

PCP bound, t is the time after addition of the pre-equilibrated halothane solution, and A_1 and A_2 are the relative sizes of receptor populations having dissociate rate constants, k_1 and k_2 , respectively. It was necessary to use higher concentrations of unlabeled PCP in order to show the effect of halothane on the PCP dissociation kinetics. The range of concentration of unlabeled PCP used (2–20 μM) did not affect the results.

RESULTS AND DISCUSSION

Administration of halothane (2.5% in nitrogen) inhibited [^3H]PCP binding by approximately 70% and reached a steady-state level within 15 min of application (Fig. 1A). Complete recovery of control levels of [^3H]PCP binding by the receptors was achieved within 15–25 min by equilibrating the incubating mixture with a stream of nitrogen without halothane. Typically, 70% recovery was reached after 5 min. Therefore, halothane inhibition of [^3H]PCP binding to the nicotinic acetylcholine receptor was reversible.

The inhibition of [^3H]PCP binding by halothane was also concentration dependent (Fig. 1B). By fitting the data to a curve using the logistic equation [21], the average IC_{50} (the concentration of halothane that inhibited one-half of [^3H]PCP binding in the control) was $2.2 \pm 0.3\%$ halothane (\pm SD; $N = 7$) at 25° . The Cheng-Prusoff correction [22] from the IC_{50} value was minimal and gave a K_d value of 2.2% halothane.

Equilibrium binding assays revealed competitive binding between halothane and [^3H]PCP at the PCP site. Assays were carried out at increasing concentrations of [^3H]PCP with a given percentage

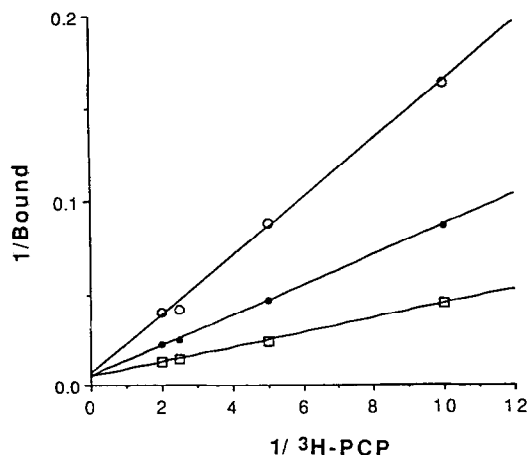


Fig. 2. Effect of halothane on the binding of $[^3\text{H}]\text{PCP}$ assayed as a function of $[^3\text{H}]\text{PCP}$ concentrations (μM). The results were analyzed by a double-reciprocal plot of $1/\text{bound}$ vs $1/[^3\text{H}]\text{PCP}$. Each linear regression fitted line represents an experiment at a constant halothane concentration. In this figure, the lines are, from top to bottom, 4.25% (\circ), 2.53% (\bullet), and 0% (\square) atm halothane, respectively.

of halothane (Fig. 2). In double-reciprocal plots, the fitted lines intersected at a single point on the Y-axis, indicating a competitive interaction between $[^3\text{H}]\text{PCP}$ and halothane [23].

The influence of halothane on the dissociation kinetics of $[^3\text{H}]\text{PCP}$ from the acetylcholine receptor is shown by a plot of percent $[^3\text{H}]\text{PCP}$ bound versus time following the addition of 200- to 2000-fold non-radioactive PCP and appropriate concentrations of halothane. To ascertain that the order of addition of halothane was not a factor in our results, halothane was pre-equilibrated with the receptor preparation or added simultaneously with the non-radioactive PCP. The results showed that the order of addition of halothane did not influence the $[^3\text{H}]\text{PCP}$ dissociation rate, suggesting a rapid halothane on-rate relative to the time scale of our experiment. With increasing concentrations of halothane (Fig. 3A), the dissociation times progressively decreased from the control half-time of 1.8 min in the absence of halothane to a value of 0.3 min in the presence of 2.5% halothane. Using the $[^3\text{H}]\text{PCP}$ dissociation half-time value as a measure of receptor response, a concentration-response curve was constructed (Fig. 3B). A non-linear curve-fit of the points yielded a 50% response concentration (EC_{50}) of 0.8% halothane at 25° . The important aspect of this finding is that, at concentrations as low as 0.8%, halothane had a significant effect on the receptor protein as determined by kinetic measurements. It has been determined that 0.8% halothane at 25° is equivalent to approximately 1.6% halothane at 37° [24]; this halothane concentration is approximately twice the minimum alveolar concentration (MAC) required to inhibit response to surgical incision in 50% of patients [25].

Data from equilibrium binding assays and PCP

dissociation rate experiments can be accommodated by a two-site model of halothane binding to the nicotinic acetylcholine receptor. Under equilibrium conditions at a range of 2.2% atm halothane, halothane competitively inhibited PCP binding to the high-affinity PCP site in the receptor, suggesting an overlap between the PCP and halothane binding sites at a range of 2.2% halothane. At lower concentrations in the range of 0.8% atm halothane, halothane promoted an increase in the dissociation rate of bound PCP from the receptor, suggesting the possibility of a second halothane binding site that is coupled allosterically to the high-affinity PCP site. Occupation of this second site increases the rate of dissociation of PCP from the receptor. Where is this halothane site? General anesthetics certainly are able to penetrate the lipid bilayer. However, general anesthetics at anesthetic concentrations were shown in EPR studies not to affect the lipid/protein interface significantly [26, 27]. Therefore, although halothane does associate with membrane lipids, the effect that we have observed is most likely not mediated by lipids. We suggest that halothane acts directly on the receptor protein.

The substantial change in $[^3\text{H}]\text{PCP}$ dissociation kinetics (a 50% decrease in dissociation half-time) induced by 0.8% halothane in the absence of a proportional change on equilibrium binding suggests two different possible interpretations. Although no global structural changes in the nicotinic acetylcholine receptor at this halothane concentration range have been reported, regional changes may still occur in protein structure at the vicinity of the halothane binding site [28]. Figure 4 is a schematic illustration of a possible model that incorporates some of these concepts. In Fig. 4B, the model illustrates how halothane may affect PCP binding allosterically and yet show competitive inhibition of PCP at a higher halothane concentration, as shown in Fig. 4C.

We have shown previously that the hexyl ring of the PCP binds to a highly hydrophobic site (with a dielectric constant of 2–3 units) in the acetylcholine receptor [29], possibly in a crevice between the receptor subunits [29, 30]. There is also evidence that these sites are accessible to uncharged anesthetics in the lipid component of the membrane [31]. Thus, it may be possible for halothane in the lipid membrane to be in equilibrium with halothane in these hydrophobic protein sites.

A possible location for the second halothane binding site is in the region immediately adjacent to the PCP site in the same hydrophobic crevice (e.g. Figure 4B). There is evidence that non-polar residues larger than PCP can be accommodated in the PCP site [12]. Thus, small, hydrophobic molecules such as halothane could fit adjacent to, but without overlapping, the PCP binding site. By binding to the receptor adjacent to the PCP site, halothane may promote dissociation of PCP from its binding site. This possibility is analogous to the type II interaction suggested by Monod *et al.* [28] where ligands do not overlap in their binding but are close enough to interact with each other. Our proposed model relies on a hydrophobic site of limited capacity. Based on this model, we predict that halothane will be unable to affect the kinetics of molecules (PCP analogs)

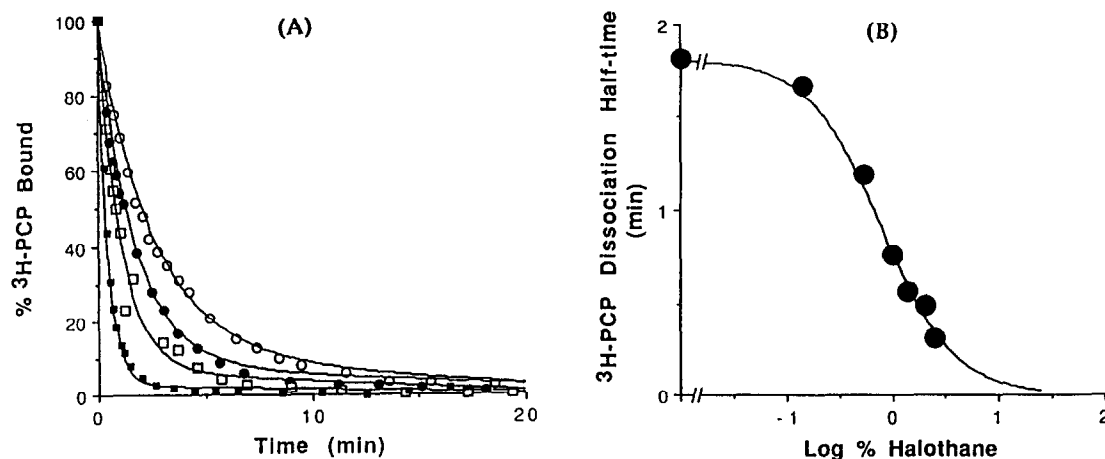


Fig. 3. Effect of halothane on the dissociation kinetics from the nicotinic acetylcholine receptor. (A) The data points at 0% (○), 0.5% (●), 1% (□), and 2% (■) halothane and their corresponding fitted curves are represented from top to bottom, respectively. (For purposes of clarity, additional graphs are not shown.) In each fitted curve, the Y-value is represented by 80–85% of the receptors having a single rate constant and a remaining population of receptors having a much smaller rate constant. (The smaller population is believed to represent inactive receptors and is not dealt with in this paper.) (B) Half-time value for each experiment was plotted against the halothane concentration for that experiment on a semilog plot in the form of a concentration–response relationship. The data points were fitted to a logistic equation [22] yielding an EC_{50} of 0.8% halothane. The dissociation half-time ($T_{1/2}$) was calculated from the rate constant (k_1) representing 80–85% of the receptors by the equation $T_{1/2} = \ln 2/k$. The average half-time for the control was 1.8 ± 0.24 min (\pm SD; $N = 8$).

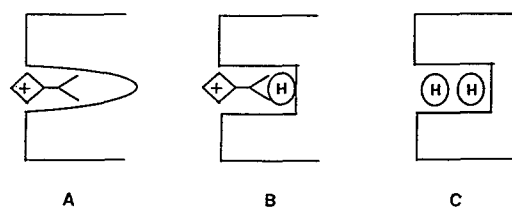


Fig. 4. Schematic illustration of the two-site model of halothane binding to the nicotinic acetylcholine receptor. The circles represent halothane molecules and the diamonds represent the positively charged PCP. (A) Illustrates the binding of a positively charged PCP at the polar-apolar interphase of the hydrophobic PCP site. (B) Binding of a halothane molecule at a neighboring site to the PCP molecule results in changes that affect the PCP dissociation kinetics. At 0.8% atm halothane, the dissociation rate constant (off rate) for PCP is doubled. (C) At 2.2% atm halothane, 50% of the bound [³H]PCP molecule is competitively displaced. (This figure is adapted from a similar figure in Ref. 28.)

sufficiently larger than PCP, which bind at the high-affinity PCP site. We further predict that halothane will retain its ability to inhibit competitively the binding of such larger PCP-analog molecules at the high-affinity PCP site.

A second interpretation of the results is to assume a single halothane binding site distinct from the PCP site in the nicotinic acetylcholine receptor. In such a model, halothane would affect [³H]PCP binding non-competitively by an allosteric mechanism.

Although a halothane-induced increase in dissociation rate of PCP would be consistent with this model, the EC_{50} at 0.8% atm for the halothane-induced dissociation rate increase is too low to account for the 50% inhibition of [³H]PCP binding at 2.2% atm halothane. In order for the single site model to account for the data, one would need to argue that both association (k_{on}) and dissociation (k_{off}) kinetics of [³H]PCP are affected by halothane, but disproportionately. Thus, the relative shift in the kinetics gives rise to a gradual change in affinity ($K_d = k_{off}/k_{on}$) of the receptor for [³H]PCP, and at 2.2% halothane, the affinity for [³H]PCP binding to the receptor becomes half that of the control. The exact mechanism of such an effect is not established. However, it can be the result of an improved diffusion path for both the on- and off-reactions.

Although we favor the former interpretation, the latter interpretation is not ruled out. However, in either case, a small hydrophobic ligand such as halothane is clearly shown to affect the kinetics of [³H]PCP binding at an allosteric site in the nicotinic acetylcholine receptor. This result could suggest a conformational change in a receptor protein at clinically significant anesthetic concentrations.

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