MOLECULAR PHARMACOLOGY, 20:498–505

Allosteric Effects of Volatile Anesthetics on the Membrane-Bound Acetylcholine Receptor Protein

I. Stabilization of the High-Affinity State

ANTHONY P. YOUNG AND DAVID S. SIGMAN

Department of Biological Chemistry, School of Medicine, and Molecular Biology Institute, University of California at Los Angeles, Los Angeles, California 90024

Received December 19, 1980; Accepted July 10, 1981

SUMMARY

The volatile anesthetics halothane, chloroform, and ether preferentially stabilize the membrane-bound acetylcholine receptor protein prepared from the marine ray *Torpedo californica* in a conformational form which binds agonists with high affinity. Since these anesthetics do not require the presence of the cationic ligand to facilitate the transition from the low-affinity state to the high-affinity state, these agents can be considered as positive heterotropic effectors of the acetylcholine receptor protein. Aliphatic alcohols, which have well-defined physiological effects at neuromuscular junctions, also stabilize the high-affinity form of the acetylcholine receptor protein *in vitro*. The effectiveness of the primary alcohols and the anesthetics in stabilizing the tightly binding form correlates with their respective membrane/buffer partition coefficients, emphasizing the importance of these perturbants' hydrophobicity in altering the structure of this membrane-bound protein. If the high-affinity form of the receptor protein is the manifestation *in vitro* of the desensitized receptor *in vivo*, this heterotropic effect could explain the depression of synaptic transmission by volatile anesthetics. By increasing the concentration of desensitized or refractory receptors at crucial central nervous system synapses, volatile anesthetics could block depolarization of postsynaptic membranes by neurotransmitters.

INTRODUCTION

Electrophysiological studies have indicated that volatile anesthetics impair synaptic transmission at the vertebrate neuromuscular junction by affecting postsynaptic membranes (1–4). In an effort to understand the biochemical basis of this phenomenon, the effects of volatile anesthetics on the membrane-bound AcChR[1] protein isolated from the electric organs of *Torpedo californica* were examined (5). We found that volatile anesthetics greatly facilitated a ligand-induced conformational transition which increases the affinity of the AcChR for agonists, such as carb by a factor of 100–1000. Because this high-affinity form of the AcChR is most likely an *in vitro* correlate of the desensitized or inactivated state observed in electrophysiological experiments (5–10), our results suggested that volatile anesthetics could affect synaptic transmission either by enhancing the rate of the ligand-induced conformational change or by stabilizing the desensitized form of the AcChR relative to the resting state of low affinity for agonists (5).

The experiments outlined here have been designed to determine the relative significance of these two mechanisms. The conformational change has been monitored by the increased inhibition of the rate of ^{125}I-labeled α-bungarotoxin ([^{125}I]α-Bgt) binding to the AcChR by the agonist carb after pretreatment with carb and/or the organic perturbant. In the micromolar concentration range, carb does not affect the binding of [^{125}I]α-Bgt to the resting or initial state of the AcChR. However, when the AcChR undergoes its conformation transition to the tightly binding form, the same concentration of carb effectively blocks [^{125}I]α-Bgt binding. Therefore the relative concentration of the tightly binding conformational state and the loosely binding conformational state of the AcChR can be readily assessed by studying the kinetics of toxin binding in the presence of 1 μM carb (6–8).

The central observation of this paper is that the volatile anesthetics halothane and chloroform act as heterotropic effectors of the AcChR in its membrane-associated form and preferentially stabilize the tightly binding conformational state of the receptor. This conclusion is based on the demonstration that pretreatment of mem-

This research was supported by United States Public Health Service Grant 21199.

[1] The abbreviations used are: AcChR, acetylcholine receptor: carb, carbamylcholine; α-Bgt, α-bungarotoxin; mepcs, miniature end-plate currents.

0026-895X/81/030498-08$02.00/0

brane fragments with the anesthetics convert the resting state AcChR into the tightly binding form in the absence of agonist. These organic perturbants also enhance the rate of the ligand-induced conformational change by decreasing the concentration of agonist required for the conversion to proceed at 50% of its maximal rate. The anesthetics do not affect the magnitude of the first-order rate constant for the conversion. The aliphatic alcohols 1-octanol, 1-butanol, and ethanol also facilitate conversion of the AcChR to the high-affinity state. Correlations with electrophysiological data indicate that 1-octanol and 1-butanol might also act as positive heterotropic effectors of the AcChR. The mechanism by which ethanol facilitates conversion to the high-affinity state is not certain.

EXPERIMENTAL PROCEDURE

Materials and methods. The following materials were obtained commercially: carb, phenylmethylsulfonylfluoride, Triton X-100, and once-recrystallized bovine serum albumin (Sigma Chemical Company, St. Louis, Mo.); lactoperoxidase (EC 1.11.1.7) (Calbiochem, San Diego, Calif.); Na^{125}I (New England Nuclear Corporation, Boston, Mass., or Amersham Corporation, Arlington Heights, Ill.); DEAE-81 filter discs (Whatman Inc., Clifton, N. J.); halothane, containing 0.01% thymol (Halocarbon Laboratories, Hackensack, N. J.); analytical grade chloroform, diethyl ether, and 1-butanol (Mallinckrodt Inc., St. Louis, Mo.); analytical grade 1-octanol (Baker Chemical Company, Phillipsburg, N. J.); 100% Gold Shield ethanol (ICN, Cleveland, Ohio); and *Bungarus multicinctus* lyophylized venom (Miami Serpentarium, Miami, Fla.). *Torpedo californica* were obtained live from Pacific Biomarine (Venice, Calif.).

α-Bgt was purified by ion exchange chromatography (CM-52 (Whatman)) (11) and gel filtration [Sephadex G-50 fine (Pharmacia Fine Chemicals, Piscataway, N. J.)] to homogeneity.

The [^{125}I]α-Bgt was prepared using lactoperoxidase and purified using Sephadex G-50 fine with 0.01 M sodium phosphate (pH 7.4) and 0.05 M NaCl as the elution buffer. Batch purification of the toxin with DEAE-cellulose using this buffer was essential to eliminate background binding in the DEAE-filter disc method developed by Schmidt and Raftery (12). Membrane fragments bearing the AcChR were prepared from *T. californica* electric organs that were excised and quick-frozen in liquid nitrogen and stored at −60° as previously described (5). The toxin-binding normalities were determined using unlabeled α-Bgt as primary standard. Assays to determine the proportion of AcChR in the low-affinity state in the presence and absence of perturbants to be described below were conducted within 2–3 days of preparations of membrane fragments.

Delivery of volatile anesthetics. The volatility and low aqueous solubility of inhalational anesthetics required a simple gas flow system (13) to permit the incubation and assay of membrane fragments under defined conditions.

To produce an atmosphere of the desired volume percentage of a volatile anesthetic (Fig. 1), a saturated gaseous mixture was first generated by passing air through the liquid anesthetic using a scintered glass tube at a monitored flow rate. The saturated mixture was then diluted with air of monitored flow rate to generate an atmosphere of gas at the desired volume percentage of anesthetic. The vapor pressure of the anesthetic and the flow rates of the air permit the volume percentage of anesthetic in the final flowing gaseous mixture to be calculated by Eq. 1:

$$\frac{\text{Fraction of anesthetic in saturated solution}}{\text{fraction of air in saturated solution}} = \frac{p}{1-p} \quad (1)$$

where p is the vapor pressure of the anesthetic. If air at flow rate x is passed through the liquid anesthetic, the

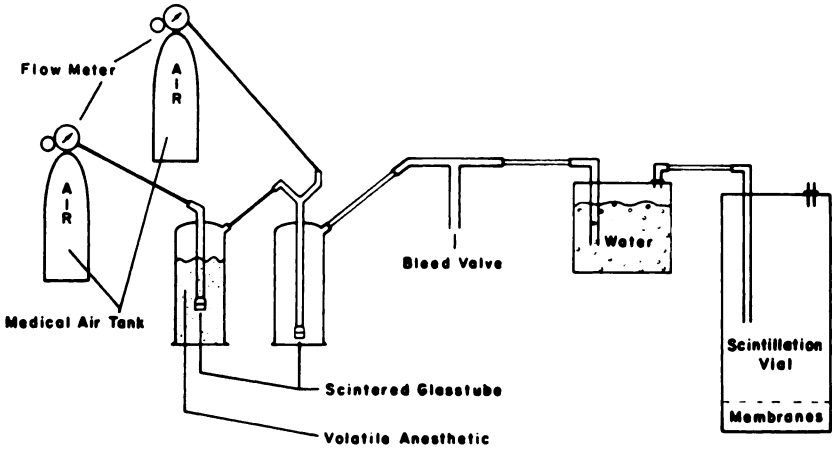


FIG. 1. *Schematic description of volatile anesthetic delivery system*

A saturated vapor of anesthetic was generated by passage of medicinal-grade air (Liquid Carbonics) through liquid anesthetic using a scintered glass tube. The effluent gaseous mixture was diluted with more medicinal-grade air. Most of the final gaseous mixture was bled to the atmosphere, but a portion was passed through a small vial of water prior to passage over the membrane solution in a 15-ml scintillation vial. The apparatus was equipped so that two membrane solutions could be equilibrated with anesthetic vapor simultaneously.

flow rate of the anesthetic gas (y) emerging with the air can be expressed by Eq. 2:

$$y = \frac{p}{1-p}(x) \tag{2}$$

Finally, when the gaseous mixture is then diluted with air of flow rate z, the volume percentage of anesthetic in the final flowing mixture is summarized by Eq. 3:

$$\text{Vol\% anesthetic} = \frac{y}{x+y+z} \times 100 \tag{3}$$

The effluent gaseous mixture was passed through a small vial of water prior to passage over the membrane solutions (1 ml of membranes in a 15-ml Beckman scintillation vial) to prevent evaporation of the membrane solution. The vapor pressures at 21° for the anesthetics halothane, chloroform, and diethyl ether were 0.334, 0.211, and 0.579 atmos, respectively.

With our experimental protocol, only the loss of anesthetic during the short time between equilibration with gaseous mixture and completion of the assay for toxin binding was a concern. Since passage of air over anesthetic-treated samples for several minutes was required to reverse the anesthetic effects, the possible loss of anesthetic during the assay could not be significant.

Kinetic measurement. The association of toxin with the AcChR can be represented by

$$A + T \xrightarrow{k} AT \tag{4}$$

where T is the concentration of uncomplexed [^{125}I]α-Bgt, A is the concentration of uncomplexed toxin binding sites, AT is the concentration of AcChR-toxin complexes, and k is the bimolecular rate constant for toxin binding. If toxin is present in excess, its concentration, T_0, does not vary when the initial rates of formation of receptor-toxin are monitored. As a result, the initial toxin binding rate, k_{fb}, defined as kT_0, can be determined from Eqs. 5 and 6.

$$AT = k(T_0)(A_0)t = k_{fb}(A_0)(t) \tag{5}$$

or

$$\frac{AT}{A_0} = \frac{A_0 - A}{A_0} = \left(1 - \frac{CPM_\infty - CPM_t}{CPM_\infty - CPM_{\text{background}}}\right) = k_{fb}(t) \tag{6}$$

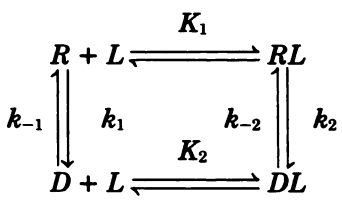
where CPM_∞ is the infinity point binding of the toxin to the filter discs expressed as counts per minute, CPM_t represents the counts bound at time, t, after initiation of the toxin-binding reaction, and $CPM_{\text{background}}$ is the background binding of the toxin to the filter discs. Four determinations of $A_0 - A/A_0$ were made between 3 and 10 sec of initiation, before 30% of the total reaction was complete.

For the kinetic runs, membranes (approximately 0.04 μM in toxin binding sites) were incubated in a closed vial, under medicinal grade air, under anesthetic or with a primary alcohol for 30 min at 21° using a thermostated water bath. Control experiments indicated that the perturbant effects described below developed within 10 min of incubation and were stable for at least 60 min (the longest time tested). Aliquots (500 μl) of the membrane solution were added to [^{125}I]α-Bgt (approximately 0.3 μM) and, where appropriate, carb (final concentration 1 μM). The membrane solution was always added in approximately 100-fold volume excess relative to the [^{125}I]α-Bgt and carb mixture. To measure initial rates of toxin binding, 100-μl aliquots were withdrawn and the reaction was quenched by addition to 10 μl of unlabeled α-Bgt (1 mg/ml or approximate 30-fold molar excess relative to the [^{125}I]α-Bgt) and 2 μl of 0.1 M carb. Infinity points for these kinetic runs were determined by incubating membranes and [^{125}I]α-Bgt for several hours followed by the addition of quenching reagents. Unless otherwise indicated, the buffer used for these experiments was composed of 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), 0.1 M NaCl, 1 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N'-N' tetraacetic acid, and 3 mM NaN_3.

The rate of the affinity conversion induced by carb was measured by pretreating the membranes with the agonist for varying times followed by addition to [^{125}I]α-Bgt and assay of the initial rate of toxin binding (6–9). This conversion is governed by a first-order rate constant which depends on agonist. At high concentrations of carb, the larger first-order rate constants were estimated from analysis of time-course of toxin binding after addition of membranes to carb and [^{125}I]α-Bgt (without preincubation) as a parallel first-order reaction (14).

RESULTS

Carb induces a reversible structural transition of AcChR to a tightly binding form (6–8). This high-affinity conformer is characterized by the inhibition of the rate of [^{125}I]α-Bgt binding to the AcChR by 1 μM carb. The two-state model for the AcChR originally proposed by Katz and Thesleff (15) to explain desensitization at the neuromuscular junction has also been used to explain this *in vitro* phenomenon. According to this model, the AcChR exists primarily in the resting or low-affinity form (R). Incubation of agonist (L) with AcChR leads to the conversion of the AcChR to the desensitized or high-affinity form of the AcChR (D). K_1 is the dissociation constant for agonist bound to a low-affinity state (RL); K_2 is the dissociation constant for agonist binding to a high-affinity state (DL); and k_1, k_{-1}, k_2, and k_{-2} are rate constants which characterize the reversible transitions.



SCHEME I

The initial rate of toxin binding to the receptor in the presence and absence of 1 μM carb can be used to estimate the fraction of total receptor (A_0) in the R form (i.e., $\bar{R}/A_0$). The ratio of the initial rate in the presence of

ligand, V_L, to that in the absence of ligand, $V_{L=0}$, is approximately equal to the fraction of the AcChR in the low-affinity form

$$\frac{V_L}{V_{L=0}} = \frac{\bar{R}}{A_0} \tag{7}$$

if the following conditions are met: (a) carb competitively inhibits the toxin binding (Fig. 2); (b) $K_1 \gg 1$ μM (Fig. 2A); (c) $K_2 \ll 1$ μM (Fig. 2B); and (d) toxin binds to unligated forms of R and D at identical rates (16). This latter property has been demonstrated in this laboratory using the D form of the receptor prepared (a) by incubation with carb, followed by dilution and assay of the initial rate of toxin binding, or (b) by prolonged storage of the AcChR at 4°. In the absence of any perturbant, the values of $\bar{R}/A_0$ for different AcChR preparations have ranged from 80% to 96% (e.g., Fig. 5 or Fig. 7), values similar to those reported in the literature (6–9, 17, 18).

Halothane effects on the AcChR. Incubation of the membrane-bound AcChR with halothane causes dramatic changes in $\bar{R}/A_0$. For example, membrane fragments incubated with 5% halothane exhibit an $\bar{R}/A_0$. of 0.31 (Fig. 3A). Since the total toxin-binding normality of the AcChR preparations is not altered by halothane pretreatment and the passage of air over the membranes does not affect the toxin-binding kinetics (Fig. 3B), the observations are not an artifact of the delivery procedure. The alteration produced by the anesthetic is observed within 10 min and remains the same after 1 hr of incubation. The effect of preincubation of halothane is readily reversed within 15 min by passage of air over anesthetic-treated samples (Fig. 3C). Moreover, if membranes are converted to the high-affinity form by carb in the presence of halothane and then diluted into a buffer containing only the anesthetic, the membrane fragments regain the value of $\bar{R}/A_0$ that characterized them after incubation with anesthetic alone in the absence of carb. The observed first-order rate constant for the regeneration of the low-affinity form is $(k_1 + k_{-1})$ (Scheme I). Its value is 0.029 sec^{-1} and 0.018 sec^{-1} in the presence and absence of 3% halothane, respectively.

Estimation of $\bar{R}/A_0$ according to Eq. 5 requires that the rate of affinity conversion of the AcChR in the presence of 1 μM carb be slower than the 3–10 sec required to measure the initial rate of toxin binding. Previously we have demonstrated that halothane and chloroform accelerate the rate of the affinity conversion induced by 0.5 μM carb. These measurements have been extended to other agonist concentrations in order to assess the extent to which the agonist-induced affinity conversion facilitated by anesthetics affect our measurements of $\bar{R}/A_0$.

An oversimplified kinetic scheme for the agonist-induced conversion which ignores some of the elements of reversibility of Scheme I (Eq. 7) is summarized in Eq. 8 (Scheme II).

$$R + L \overset{K_1}{\rightleftharpoons} RL \overset{k_{max}}{\rightarrow} DL \tag{8}$$

The observed first-order rate for the conversion, k_i, according to Scheme II, is given by Eq. 9

$$k_i = \frac{k_{max} L}{K_1 + L} \tag{9}$$

which can be converted to its double-reciprocal form, Eq. 10.

$$\frac{1}{k_i} = \frac{1}{k_{max}} + \left(\frac{K_1}{k_{max}}\right)\frac{1}{L} \tag{10}$$

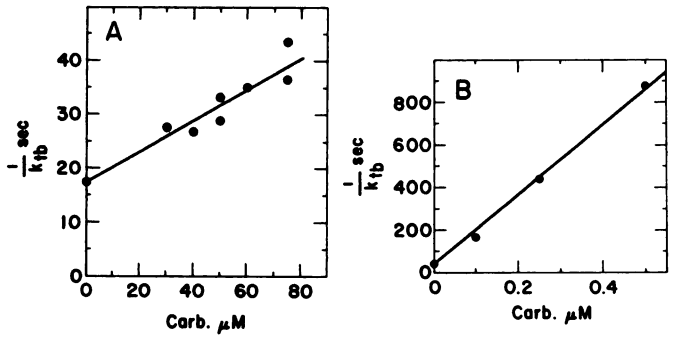


FIG. 2. *Competitive inhibition by carb of the rate of [^{125}I]α-Bgt binding*

A. Competitive inhibition by carb of the rate of [^{125}I]α-Bgt binding to the low-affinity state of the membrane-bound AcChR. Membranes (0.042 μM in toxin binding sites) were added to the indicated concentration of carb and [^{125}I]α-Bgt (0.28 μM) followed by determination of the pseudo-first order rate constant for toxin binding. The data was plotted according to the equation $1/k = 1/k_{tb} + (1/k_{tb})(L/K_2)$. K_i was 61 μM.

B. Competitive inhibition by carb of the rate of [^{125}I]α-Bgt binding to the high-affinity state of the membrane-bound AcChR. Membranes (0.031 μM in toxin binding sites) which had converted to a high-affinity state upon prolonged storage were pretreated with the indicated concentrations of carb for 30 min followed by addition to 0.21 μM [^{125}I]α-Bgt and assayed for time course of toxin binding. K_i was 0.02 μM. Similar results were obtained using AcChR converted to the high-affinity state with carb, followed by dilution to a lower carb concentration and assay for the initial rate of [^{125}I]α-Bgt binding.

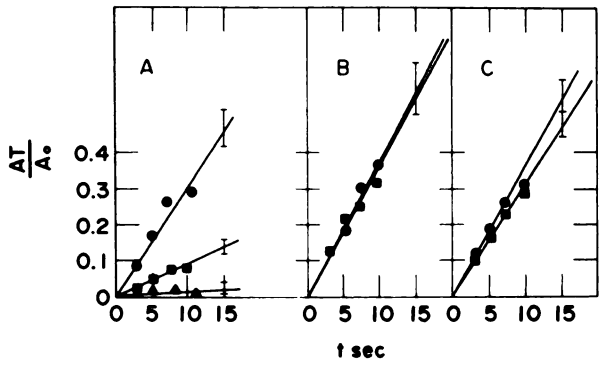


FIG. 3. *Conversion of the membrane-bound AcChR to the high-affinity state by 5% halothane*

A. Membranes (0.038 μM in toxin binding sites) were incubated for 30 min at 21° under an atmosphere of 5% halothane and added to [^{125}I]α-Bgt (0.28 μM) alone (●) or to [^{125}I]α-Bgt and 1 μM carb (■), or membranes were incubated with 1 μM carb prior to addition to [^{125}I]α-Bgt (▲) and assayed for the initial rate of toxin binding.

B. Membrane fragments were incubated under a stream of air and then added to [^{125}I]α-Bgt in the presence (■) and absence of 1 μM carb (●).

C. Membrane fragments were incubated under an atmosphere of 5% halothane followed by an additional 30 min of preincubation under a stream of air. They were then assayed with [^{125}I]α-Bgt in the presence of (■) and absence of 1 μM carb (●).

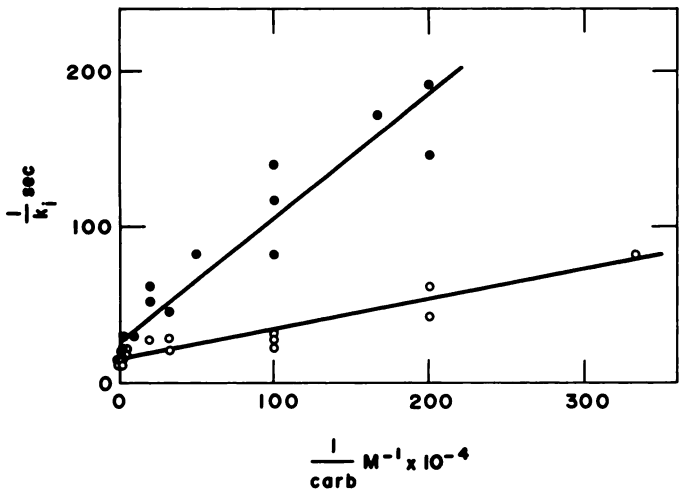


FIG. 4. *Kinetics of the carb-dependent affinity conversion in the presence and absence of 3% halothane*

First-order rates (k_i values) for the affinity conversion were obtained at the indicated carb concentrations as discussed in the text after preincubation of membranes for 30 min at 21° in the presence (○) and absence (●) of 3% halothane. The *lines* drawn are least-squares fits of the data to Eq. 10.

Although this treatment is incomplete, it provides a convenient approach to illustrate whether anesthetics preferentially affect either k_{max} or K_1. The data of Fig. 4 demonstrate that 3% halothane primarily accelerates the rate of ligand-induced conversion by decreasing the concentration of carb necessary to achieve conversion at 50% of the maximal rate. The maximal rate is relatively unaffected, changing from 0.04 to 0.07 sec^{-1} in the absence and presence of 3% halothane, respectively. Quantitative treatments, incorporating all possible reversible steps, have shown that k_2 from Scheme I is 0.07 sec^{-1}, using untreated membranes as well as those incubated with 1% chloroform or 3% halothane (19).[2] Conversion at this anesthetic-insensitive limiting rate ($t_{1/2}$ = 10 sec) cannot account for the values of $\bar{R}/A_0$ shown in Figs. 5 and 6 since the toxin-binding rates used to estimate $\bar{R}/A_0$ were calculated from time points obtained as soon as 3 sec after the addition of carb and toxin to the AcChR-bearing membranes (cf. Fig. 3A). Therefore, halothane acts as a heterotropic effector and stabilizes the high-affinity conformer of the AcChR in the absence of carb.

Additional evidence for the perturbation of receptor structure by anesthetics in the absence of agonists is supported by the biphasic toxin kinetics observed in the absence of agonist after membrane fragments have been incubated with volatile anesthetics (2).

The ratio $\bar{R}/A_0$ of AcChR is maximally sensitive to halothane in the 3–5% concentration range of the anesthetic. The severe concentration dependence is consistent with several molecules of halothane stabilizing the D form relative to the R form. Volatile anesthetics are not unique in their ability to stabilize the D form. This property is also shared by certain local anesthetics (17, 18, 20) as well as calcium ion, which decrease the $\bar{R}/A_0$ to 0.63 when 1 mM calcium chloride replaces ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid in the buffer. Calcium ion and halothane must stabilize the D form at different loci because the effects of the two agents are synergistic.

[2] The value for k_2 reported here using Ca^{2+}-free buffers is 2-fold slower than that observed by Quast *et al.* (9) using membrane preparations incubated in a buffer containing calcium. However, it is 5- to 6-fold faster than that observed by Weiland *et al.* (20) using a Ca^{2+}-free buffer different from ours. Differences in observed rates for the affinity conversion therefore reflect factors other than just the presence or absence of Ca^{2+}.

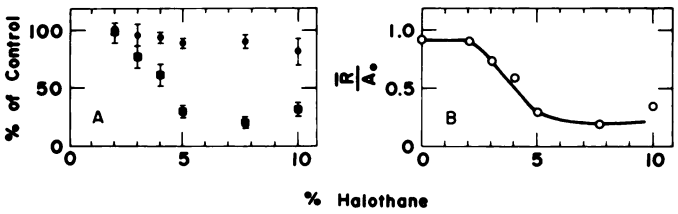


FIG. 5. *Concentration dependence of the conversion of the AcChR to the high-affinity state by halothane*

A. Membranes (0.038 μM in toxin binding sites) were pretreated with halothane for 30 min at 21°. Aliquots were removed and added to [^{125}I] α-Bgt (0.28 μM) alone (●) or added to [^{125}I]α-Bgt and 1 μM carb (■) and initial rates of toxin binding were assayed. The data are means of three to six determinations of the initial rates and are expressed as percentage of the control rate obtained in the absence of halothane. *Error bars* indicate standard deviations. Control rates for ● and ■ were 3.57 × 10^{-2} sec^{-1} and 3.38 × 10^{-2} sec^{-1}, respectively.

B. The fraction of low-affinity state receptor ($\bar{R}/A_0$) was calculated as described in the text as a function of halothane concentration.

Chloroform. Other volatile anesthetics facilitate the conversion of the AcChR to its high-affinity form. Membrane fragments incubated with chloroform demonstrate comparable results (Fig. 6). In the concentration range of 1–3% chloroform, a sharp transition in $\bar{R}/A_0$ is observed. Chloroform differs in one important respect from halothane. It inhibits the association rate of α-bungarotoxin at concentrations whereby it alters $\bar{R}/A_0$ (1–3%). Nevertheless, the reduction of toxin binding by chloroform is proportionally much more substantial when as-

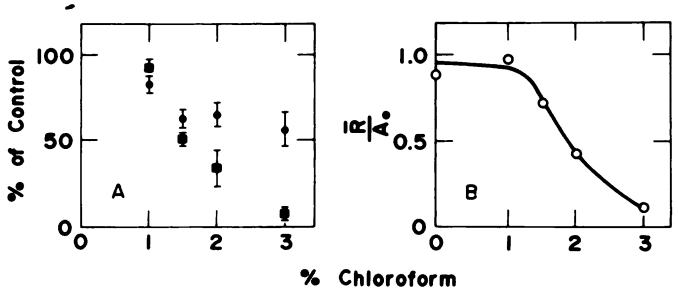


FIG. 6. *Concentration dependence of the conversion of the AcChR to the high-affinity state by chloroform*

A. Membranes were pretreated with chloroform for 30 min at 21°. Aliquots were removed and added to [^{125}I]α-Bgt alone (●) or added to [^{125}I]α-Bgt and 1 μM carb (■) followed by assay for the initial rate of toxin binding. Toxin binding site and [^{125}I]α-Bgt concentrations were 0.043 μM and 0.28 μM, respectively. The data are means of three to six determinations of the initial rate and expressed as percentage of the control rate obtained in the absence of chloroform. *Error bars* indicate standard deviations. Control rates for ● and ■ were 4.52 × 10^{-2} sec^{-1} and 4.00 × 10^{-2} sec^{-1}, respectively.

B. The fraction of low-affinity state receptor ($\bar{R}/A_0$) was calculated as a function of chloroform concentration.

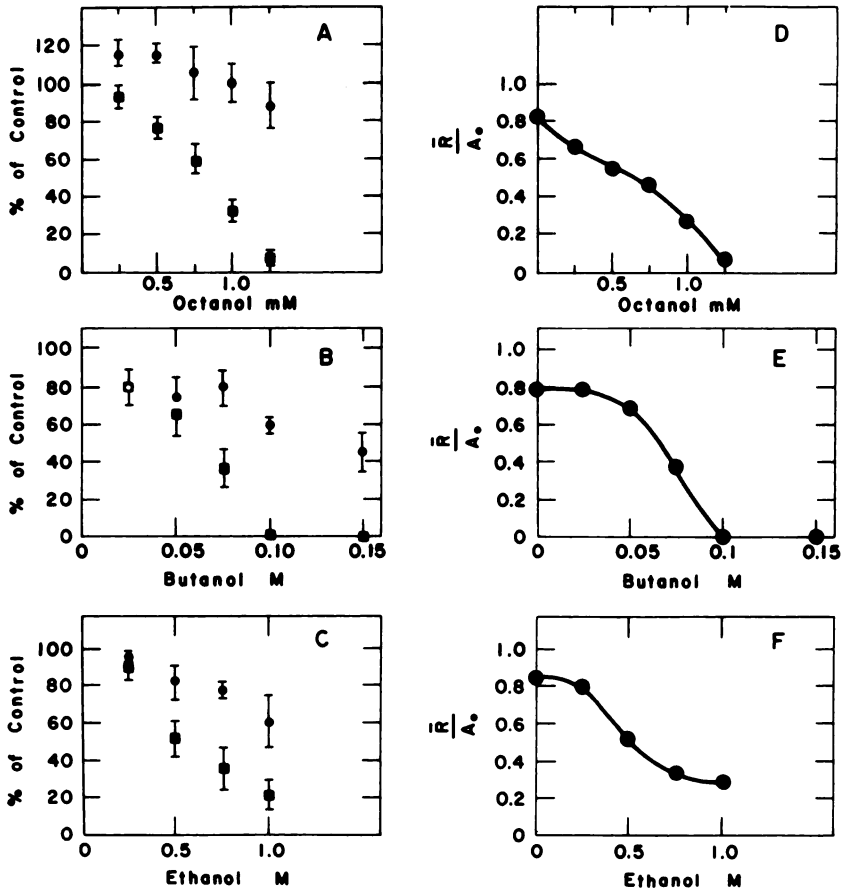


FIG. 7. *Conversion of the AcChR to the high-affinity state by primary alcohols*

A, B, and C. Membranes were pretreated with octanol (A), butanol (B), or ethanol (C) prior to addition of [^{125}I]α-Bgt alone (●) or to toxin and 1 μM carb (■) followed by assay for the initial rate of toxin binding. Toxin binding sites and [^{125}I]α-Bgt concentrations were 0.050 μM and 0.42 μM (A), 0.046 μM and 0.28 μM (B), and 0.042 μM and 0.28 μM (C), respectively. The data are means of three to six determinations and are expressed as percentage of the control rate obtained in the absence of primary alcohols. *Error bars* show the standard deviations. Control rates for ● and ■ were 5.24 and 4.28 (A), 3.49 and 2.74 (B), and 3.65 and 3.05 (C) × 10^{-2} sec^{-1}, respectively.

D, E, and F. The fraction of low-affinity state receptor ($\bar{R}/A_0$) was calculated from the data in A, B, and C as described in the text as a function of concentration of added octanol (D), butanol (E), and ethanol (F).

sayed in the presence of carb than in its absence. Calcium ion also has a synergistic effect with chloroform in the conversion of the AcChR to the high-affinity form.

The third volatile anesthetic which exhibited an effect on this affinity conversion of the receptor protein is diethyl ether. Under an atmosphere of 6% diethyl ether, substantial conversion of the membrane-bound AcChR to the high-affinity form was observed.

Aliphatic alcohols. Aliphatic alcohols have well-defined electrophysiological effects at neuromuscular junctions (22–24), and we have examined their effect on the affinity conversion *in vitro* of *Torpedo* AcChR. 1-Octanol causes a decrease in both the amplitude and time constant of decay of mepcs at the vertebrate neuromuscular junction (22). We find that incubation of *Torpedo* membranes with 0.25 mM–1.0 mM octanol for 15 min converts the AcChR to the high-affinity state (Fig. 7A). 1-Butanol causes a decrease in the amplitude of mepcs but increases their time constant of decay (23). 1-Butanol (0.025 M–0.1 M) also causes conversion to the high-affinity state (Fig. 7B). The parallel decreases in amplitude of mepcs and facilitation of conversion to the high-affinity conformer of the AcChR caused by butanol and octanol is consistent with the notion that conversion to the high-affinity state monitors receptor desensitization.

However, this parallel is weakened when comparing the effect of ethanol on the affinity conversion *in vitro* and its effect on the amplitude of mepcs. Although ethanol prolongs the duration of mepcs, it does not decrease, and, in fact, causes small increases in their amplitude (24). Fig. 7C demonstrates that ethanol (0.25 M–1.0 M) facilitates conversion to the high-affinity state, although this effect is less dramatic than that observed for octanol and butanol. The explanation for the lack of correlation between the effect of ethanol on the affinity conversion *in vitro* and its (lack of) effect on the amplitude of mepcs is not readily apparent. It is possible that *Torpedo* AcChR and AcChR at the neuromuscular junction differ in their sensitivity to ethanol. Moreover, a detailed analysis of the kinetics of the carb-induced affinity conversion has not been attempted in the presence of ethanol. Ethanol might cause an increase in the maximal rate for the affinity conversion beyond 0.07 sec^{-1}. If so, the initial rate of toxin binding in the presence of 1 μM carb and ethanol could be slow because of the rapid carb-induced conversion to the high-affinity state as opposed

to conversion by ethanol prior to the addition of carb. It is noteworthy that ethanol does produce increases in the rate of agonist-induced desensitization at the neuromuscular junction monitored by an electrophysiological assay (25).

All of the aliphatic alcohols tested produced alterations in toxin binding in the absence of carb. 1-Octanol caused small increases in this rate (Fig. 7A), whereas while 1-butanol and ethanol produced decreases at the same concentration which affected the affinity conversion (Fig. 7B and C). It is of interest that all agents tested which inhibit toxin binding in the absence of carb (halothane, chloroform, ethanol, and butanol) also decreased the rate of decay of mepcs (2, 23, 24). 1-Octanol does not inhibit toxin binding in the absence of carb when present at concentrations up to 2.5 mM, near its solubility in water, and causes an increase in the rate of decay of mepcs (22).

Correlation of kinetic effects with membrane/buffer partition coefficients. Detergent solubilization of membrane-bound AcChR yields protein with complex ligand properties suggestive of multiple noninterconverting conformational states (26). Membrane-protein interactions therefore must provide an important conformational constraint on the AcChR which might be disrupted by the hydrophobic alcohols and anesthetics utilized here. The failure of anesthetics to affect any of the toxin binding properties of the solubilized receptor either in the presence or absence of antagonist or agonist lends some support to this view (5). Correlation of the concentrations at which the affinity conversion is facilitated by the anesthetics and alcohols with their membrane/buffer partition coefficients provides additional evidence for the importance of hydrophobicity in the action of these perturbants on the conformation of the AcChR.

The membrane/buffer partition coefficients for ethanol, butanol, octanol, chloroform, and halothane are 0.14, 1.5, 152, 18.7, and 12.9, respectively (27). Aqueous concentrations of the volatile anesthetics were estimated from literature values for buffer/gas partition coefficients in the same saline buffer (27). The values are 6.8 and 1.5 for chloroform and halothane, respectively. The aqueous concentration can be calculated from the volume percentage of anesthetic in the gas phase by the following expression which considers the anesthetic-air mixture as an ideal gas:

$$\text{Molarity in aqueous phase} = \frac{\text{vol \% in gas phase}}{22.4} \times \text{buffer/gas partition coefficient}$$

The values of aqueous concentration are 5.8 mM and 2.7 mM for chloroform and halothane. Inspection of Fig. 8 reveals that, with the exception of halothane, the concentration of perturbant required for half-conversion correlates very well with the membrane/buffer partition coefficient. The *line* drawn is a least-squares fit of the alcohol data. This good correlation is consistent with the primary alcohols and chloroform affecting the conversion by a hydrophobic effect.

DISCUSSION

The volatile anesthetics chloroform and halothane are positive heterotropic allosteric effectors of the AcChR.

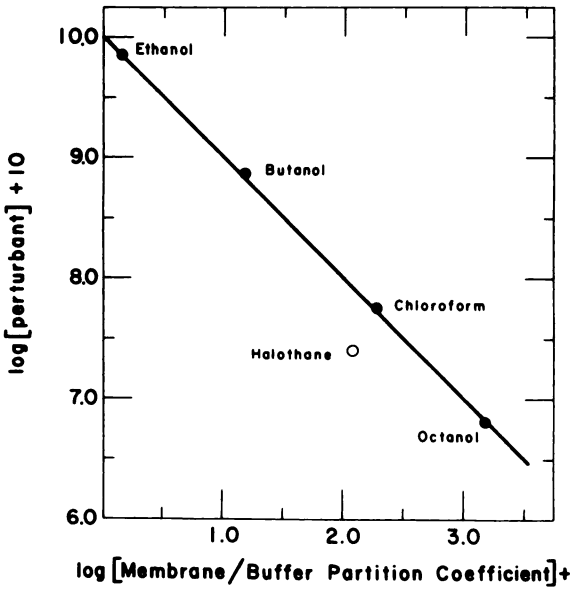


FIG. 8. *Correlation between concentration of perturbant required to convert the AcChR to the high-affinity state with membrane/buffer partition coefficients*

The log of the perturbant concentration in the aqueous phase which diminishes $\bar{R}/A_0$ to one-half of its control value is plotted against the log of the membrane/buffer partition coefficient for ethanol, butanol, octanol, chloroform, and halothane. The *line* drawn is a least-squares fit of the primary alcohol data. Perturbant concentrations were obtained from Figs. 5–8, and membrane/buffer partition coefficients were obtained as described in the text.

They stabilize the high-affinity state of the protein at the expense of the low-affinity form in the absence of agonist. Although these perturbants also enhance the rate of the ligand-induced conversion to the high-affinity form, they achieve this by decreasing the concentration of carb required for maximal conversion without altering the magnitude of the first-order rate constant. This rate is sufficiently slow so that ligand-induced conversion cannot account for the low values of $\bar{R}/A_0$ observed when receptor is preincubated with anesthetic and assayed in the presence of 1 μM carb. The aliphatic alcohols 1-octanol, 1-butanol, and 1-ethanol also affect the affinity conversion of the AcChR. Correlations with electrophysiological data suggest that octanol and butanol are acting as positive heterotropic effectors, whereas the mechanism by which ethanol affects the affinity conversion is not certain. It should be pointed out that Cohen and coworkers (18) have reported that 2-propanol facilitates conversion of *Torpedo* AcChR to the high-affinity state.

The allosteric model for volatile anesthetic action of the membrane-bound AcChR from *Torpedo* is similar to that proposed for the action of local anesthetics on this protein (17, 18, 20). Although the allosteric effects of the local anesthetics may arise from binding to an ion channel of the AcChR (17, 18), a comparable locus for volatile anesthetics on any of the subunits of the AcChR may not exist. Instead, it is possible that the allosteric effects could arise from perturbations at the protein-lipid interface resulting in the relaxation of essential conformational constraints.

If the high-affinity state of the AcChR is responsible for the desensitized postsynaptic membrane, this heter-

otropic effect by volatile anesthetics could account for their ability to block synaptic transmission at neuromuscular junctions (1–4). The concentration dependence of the anesthetic effects will be a function of the fraction of activated receptors at the synapse required to produce an action potential and the concentration of effectors (e.g., calcium ion and membrane potential) which may alter k_{-1}/k_1 (Scheme I). Since glutamate (28–30) and some γ-aminobutyric acid receptors (31) are susceptible to densensitization, it will be interesting to see whether they respond to volatile anesthetics in a manner parallel to that of the AcChR of *Torpedo* electroplax. If crucial central nervous system receptors exhibit volatile anesthetic-sensitive desensitization, the phenomena reported here may serve as a biochemical and structural model for anesthesia.

REFERENCES

1. Barker, J. L. Selective depression of postsynaptic excitation by general anesthetics, in *Molecular Mechanisms of Anesthesia* (B. Raymond Fink, ed.). Raven Press, New York, 135–153 (1975).
2. Gage, P., and O. P. Hamill. Effects of several inhalational anesthetics on the kinetics of postsynaptic conductance changes in mouse diaphragm. *Br. J. Pharmacol.* **57**:263–272 (1976).
3. Karis, J. H., A. J. Gissen, and W. L. Nastuk. The effect of volatile anesthetic agents on neuromuscular transmission. *Anesthesiology* **28**:128–134 (1967).
4. Waud, B. E., and D. R. Waud. Comparison of the effects of general anesthetics on the end-plate of skeletal muscle. *Anesthesiology* **43**:540–547 (1975).
5. Young, A. P., F. F. Brown, M. J. Halsey, and D. S. Sigman. Volatile anesthetic facilitation of *in vitro* desensitization of membrane-bound acetylcholine receptor from *Torpedo californica*. *Proc. Natl. Acad. Sci. U. S. A.* **75**:4563–4567 (1978).
6. Weber, M., T. David-Pfeuty, and J. P. Changeux. Regulation of binding properties of the nicotinic receptor protein by cholinergic ligands in membrane fragments from *Torpedo marmorata*. *Proc. Natl. Acad. Sci. U. S. A.* **72**:3443–3447 (1975).
7. Weiland, G., B. Georgia, V. T. Wee, C. F. Chignell, and P. Taylor. Ligand interactions with cholinergic receptor-enriched membranes from *Torpedo*: influence of agonist exposure on receptor properties. *Mol. Pharmacol.* **12**:1091–1105 (1976).
8. Lee, T., V. Witzemann, M. Schimerlik, and M. A. Raftery. Cholinergic ligand-induced affinity changes in *Torpedo californica* acetylcholine receptor. *Arch. Biochem. Biophys.* **183**:57–63 (1977).
9. Quast, U., M. Schimerlik, T. Lee, V. Witzemann, S. Blanchard, and M. A. Raftery. Ligand-induced conformational changes in *Torpedo californica* membrane-bound acetylcholine receptor. *Biochemistry* **17**:2405–2414 (1978).
10. Sine, S., and P. Taylor. Functional consequences of agonist mediated state transitions of the cholinergic receptor. *J. Biol. Chem.* **254**:3315–3326 (1979).
11. Clark, D. G., D. D. Macmurchie, E. Elliott, R. G. Wolcott, A. M. Landel, and M. A. Raftery. Elapid neurotoxin purification, characterization and immunochemical studies of α-bungarotoxin. *Biochemistry* **11**:1663–1668 (1972).
12. Schmidt, J., and M. A. Raftery. A simple assay for the study of solubilized acetylcholine receptors. *Anal. Biochem.* **52**:349–354 (1973).
13. Sadove, M. D., and V. E. Wallace. *Halothane*. F. A. Davis, Philadelphia (1962).
14. Frost, A. A., and R. G. Pearson. *Kinetics and Mechanism*. John Wiley, New York (1961).
15. Katz, B., and S. Thesleff. A study of the "desensitization" produced by acetylcholine at the motor end-plate. *J. Physiol (Lond.)* **138**:63–80 (1957).
16. Blanchard, S. G., U. Quast, K. Reed, T. Lee, M. Schimerlik, R. Vandlen, T. Claudio, C. D. Strader, H. P. H. Moore, and M. A. Raftery. Interaction of ^{125}I-α-bungarotoxin with acetylcholine receptor from Torpedo californica. *Biochemistry* **18**:1875–1883, (1979).
17. Heidmann, T., and J. P. Changeux. Fast kinetic studies on the allosteric interactions between acetylcholine receptor and local anesthetic binding sites. *Eur. J. Biochem.* **94**:281–296 (1979).
18. Cohen, J. B., N. D. Boyd, and N. S. Shera. Interactions of anesthetics with nicotinic postsynaptic membranes isolated from *Torpedo* electric tissue, in *Molecular Mechanisms of Anesthesia* (B. R. Fink, ed.), Vol. I. Raven Press, New York 165–174 (1980).
19. Young, A. P. Allosteric effects of volatile anesthetics on the acetylcholine receptor protein. thesis, Ph.D. Department of Biological Chemistry, UCLA (1979).
20. Weiland, G., B. Georgia, S. Lappi, C. F. Chignell, and P. Taylor. Kinetics of agonist-mediated transitions in state of the cholinergic receptor. *J. Biol. Chem.* **252**:7648–7656 (1977).
21. Young, A. P., J. R. Oshiki, and D. S. Sigman. Allosteric effects of volatile anesthetics on the membrane-bound acetylcholine receptor protein. II. Alteration of α-bungarotoxin binding kinetics. *Mol. Pharmacol.* **20**:506–510 (1981).
22. Gage, P. W., R. N. McBurney, and D. Van Helden. End-plate currents are shortened by octanol: possible role of membrane lipid. *Life Sci.* **14**:2277–2283 (1974).
23. Wann, K., M. Ashford, and A. Macdonald. The effect of alcohol anesthetics and high hydrostatic pressure on spontaneous activity of the neuromuscular junction, in *Molecular Mechanism of Anesthesia* (B. Raymond Fink, ed.). Raven Press, New York 361–371 (1980).
24. Gage, P. W., R. N. McBurney, and G. J. Schneider. Effects of some aliphatic alcohols on the conductance change caused by a quantum of AcCh at the toad end-plate. *J. Physiol. (Lond.)* **244**:409–429 (1975).
25. Anwyl, R., and I. Narahashi. Comparison of desensitization and time-dependent block of the acetylcholine receptor responses by chlorpromazine, cytochalasin B, Triton X-100 and other agents. *Br. J. Pharmacol.* **69**:99–106 (1980).
26. Heidmann, T., and J. P. Changeux. Structural and functional properties of the acetylcholine receptor protein in its purified and membrane-bound states. *Annu. Rev. Biochem.* **47**:371–441 (1978).
27. Seeman, P. The membrane action of anesthetics and tranquilizers. *Pharmacol. Rev.* **24**:583–655 (1972).
28. Dudel, J. Potentiation and desensitization after glutamate induced postsynaptic currents at the crayfish neuromuscular junction. *Pfluegers Arch. Gesamte Physiol.* **356**:317–327 (1975).
29. Dudel, J. Kinetics of postsynaptic action of glutamate pulses applied iontophoretically through high resistance micro-pipettes. *Pfluegers Arch. Gesamte Physiol.* **356**:329–346 (1975).
30. Takeuchi, A., and N. Takeuchi. The effect on crayfish muscle of iontophoretically applied glutamate. *J. Physiol. (Lond.)* **170**:296–317 (1964).
31. Epstein, R., and H. Grundfest. Desensitization of gamma-aminobutyric acid (GABA) receptors in muscle fibers of the crab *Cancer borealis*. *J. Gen. Physiol.* **56**:33–45 (1970).

Send reprint requests to: Dr. David S. Sigman, Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, Calif. 90024.