Desensitization of Membrane-Bound *Torpedo* Acetylcholine Receptor by Amine Noncompetitive Antagonists and Aliphatic Alcohols: Studies of [^{3}H]Acetylcholine Binding and $^{22}Na^+$ Ion Fluxes†

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ABSTRACT: Measurements of the kinetics of binding of [^{3}H]acetylcholine ([^{3}H]AcCh) to membrane-bound nicotinic AcCh receptors from *Torpedo* electric tissue have been used to characterize the effects of a series of amine and alcohol noncompetitive antagonists on receptor conformational equilibria. The receptor exists in multiple, interconvertible conformations distinguished by agonist binding affinity. In the absence of cholinergic ligands, certain aromatic amines including proadifen, dimethisoquin, and lidocaine, as well as propanol and butanol, produce a dose-dependent increase in the fraction of receptors (f) in a high-affinity conformation from a value of $f_{max} \sim 0.17$ in the absence of drug to $f_{max} \sim 0.9$. Not all noncompetitive antagonists produce that same value of f_{max}. For histrionicotoxin (HTX), $f_{max} \sim 0.3$, and the aromatic amine adiphenine did not alter f while tetracaine actually decreased f to 0.1. The high-affinity receptor conformation stabilized by noncompetitive antagonists was characterized by (1) the rate constant (k_{rec}) for receptor reisomerization upon removal of stabilizing ligand and (2) the rate constant (k_{dis}) for dissociation of [^{3}H]AcCh–receptor complexes. On the basis of these criteria, the high-affinity receptor conformation stabilized by amine and alcohol noncompetitive blockers is the same as that stabilized by agonist. At 4 °C, $k_{rec} = (2.2 \pm 0.2) \times 10^{-3}$ s^{-1} and $k_{dis} = 4 \times 10^{-2}$ s^{-1}. Since HTX and adiphenine produced only a small conformational perturbation, their effects on the actions of proadifen and 2-propanol were examined. HTX and adiphenine antagonized the conformational perturbation caused by proadifen, while mixtures of HTX and 2-propanol produced additive effects. Effects of noncompetitive blockers were also assayed in terms of the inhibition of agonist-induced efflux of $^{22}Na^+$ from *Torpedo* vesicles. Exposure to proadifen in the absence of agonist produced a reversible inhibition (desensitization) of the flux response, and recovery from desensitization occurred at the same rate as the reisomerization from the high-affinity receptor state. HTX, which did not cause desensitization of the flux response, reduced the desensitization by proadifen. These results are compatible with the hypothesis that certain noncompetitive antagonists modify receptor function by stabilizing the same high-affinity (desensitized) conformation that is stabilized by agonist. This is accomplished either as a consequence of binding to the distinct allosteric site or by an alternate, presumably nonspecific mechanism. However, not all noncompetitive antagonists that bind to that specific site stabilize the same high-affinity conformation.

The binding of acetylcholine (AcCh)[1] by the nicotinic cholinergic receptor results in the formation of a transmembrane channel for cations. This permeability response, which occurs within a fraction of a millisecond, is not the result of the equilibrium binding of AcCh, since exposure to a constant concentration of AcCh for seconds or longer results in a reversible decline (desensitization) in cation permeability [for a review, see Peper et al. (1982)]. To account for desensitization at the vertebrate neuromuscular junction, Katz & Thesleff (1957) and Rang & Ritter (1970) proposed a two-state cyclic model:

$$\begin{array}{ccc} D + R_{Lo} & \rightleftharpoons & DR_{Lo} \\ \downarrow\uparrow & & \downarrow\uparrow \\ D + R_{Hi} & \rightleftharpoons & DR_{Hi} \end{array} \quad (1)$$

The receptor contained a single class of AcCh binding sites that exists in the absence of agonist in a conformation (R_{Lo}) binding agonist with low affinity that is associated with channel activation and in a conformation (R_{Hi}) binding agonist with high affinity that is functionally desensitized. Studies of the kinetics of binding of agonists both in muscle cell lines (Sine & Taylor, 1980, 1981) and in nicotinic postsynaptic membranes isolated from *Torpedo* electric tissue (Weiland et al., 1977; Heidmann & Changeux, 1979a,b, 1980; Boyd & Cohen, 1980a,b) provide direct evidence for this general model, although at least in *Torpedo* there must be multiple low-affinity conformations (Neubig & Cohen, 1980). For *Torpedo* membranes, direct measurement of the kinetics of binding of [^{3}H]AcCh (Boyd & Cohen, 1980a,b) indicates that in the absence of agonist about 80% of the sites exist in a low-affinity conformation, and the remaining sites exist in a conformation binding AcCh with high affinity (K_d = 2 nM) and with a bimolecular association rate constant ($k_+ = 3 \times 10^7$ M^{-1} s^{-1}) close to that expected for a diffusion-controlled reaction. Direct comparison of the rate of formation of liganded high-affinity receptors with the rate of ion flux desensitization established that it is an inactive (desensitized) receptor conformation (Neubig et al., 1982).

Noncompetitive antagonists are by definition compounds that block the action of AcCh without preventing its binding. Such compounds would include those that stabilized a desensitized receptor conformation, whether as a result of a nonspecific perturbation of membrane structure or as a con-

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[1] Abbreviations: AcCh, acetylcholine; α-BgTx, α-bungarotoxin; Carb, carbamylcholine; H_{12}-HTX, *dl*-perhydrohistrionicotoxin; PTA, phenyltrimethylammonium; TPS, *Torpedo* physiological saline (250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM NaP_i, pH 7); DFP, diisopropyl phosphorofluoridate.

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sequence of binding to a specific site. Noncompetitive antagonists might also act by preventing the coupling between agonist binding and channel opening or by blocking the structure of the ion channel itself. In electrophysiological studies at the vertebrate neuromuscular junction, a variety of aromatic amines as well as structurally unrelated compounds including alcohols, general anesthetics, fatty acids, and detergents have been shown to act as noncompetitive antagonists either by affecting desensitization or by altering ion transport through open channels [for a review, see Spivak & Albuquerque (1982)].

In studies utilizing membrane-bound receptor from *Torpedo* electric tissue, it has been possible to examine effects of noncompetitive antagonists on receptor conformational equilibria (as defined by agonist binding affinity) and also to use radiolabeled noncompetitive antagonists to define specific binding sites and nonspecific partitioning [for a review, see Changeux (1981) and Conti-Tronconi & Raftery (1982)]. Noncompetitive antagonists appear to act as allosteric regulators of receptor conformational equilibria. Many aromatic amines (Cohen et al., 1974; Krodel et al., 1979), as well as aliphatic alcohols (Sauter et al., 1980) and detergents such as Triton X-100 (Brisson et al., 1975), increase the affinity with which [^{3}H]AcCh is bound at equilibrium. Analysis of the kinetics of binding of a fluorescent cholinergic agonist indicates that in the absence of agonist, aromatic amines such as proadifen and dimethisoquin increase the amount of receptor in a conformation with high affinity for agonist (Heidmann & Changeux, 1979b; Heidmann et al., 1983). In studies utilizing radiolabeled α-neurotoxins, aliphatic alcohols were seen to have similar effects on conformational equilibria of *Torpedo* receptors (Young & Sigman, 1981) as did aromatic amines interacting with nicotinic receptors in the muscle cell line BC3H-1 (Sine & Taylor, 1982). In addition, in the presence of agonist, amine and alcohol noncompetitive antagonists accelerate the transition of *Torpedo* receptors from the low- to the high-affinity conformation (Weiland et al., 1977; Heidmann & Changeux, 1979b; Neubig et al., 1979; Young & Sigman, 1982) although the aromatic amine tetracaine appears as an exception (Blanchard et al., 1979).

Direct determination of the binding by *Torpedo* postsynaptic membranes of potent amine noncompetitive antagonists, including [^{14}C]meproadifen (Krodel et al., 1979), [^{3}H]perhydrohistrionicotoxin (Eldefrawi et al., 1977, 1978, 1980b; Elliot & Raftery, 1977, 1979), and [^{3}H]phencyclidine (Kloog et al., 1980; Eldefrawi et al., 1980a; Oswald & Changeux, 1981), established the existence of a specific binding site distinct from the site of binding of AcCh and competitive antagonists. Although initial estimates of the number of amine antagonist sites per acetylcholine site varied from 0.25 to 2, recent studies focusing on the question of site stoichiometry indicate the presence of one noncompetitive antagonist high-affinity site per two AcCh sites, i.e., one allosteric site per receptor monomer (Heidmann et al., 1983; Medynski, 1983; Strnad & Cohen, 1983).

In this report, we examine a group of noncompetitive antagonists in terms of their effects on receptor conformational equilibria as defined by [^{3}H]AcCh binding and the agonist-induced $^{22}Na^+$ flux. The primary focus was to characterize perturbations of receptor conformational equilibria in the absence of agonist. Drugs were chosen for study from two classes: aromatic amines that bind to the allosteric site on the acetylcholine receptor and aliphatic alcohols that might act as nonspecific membrane perturbants. Amine noncompetitive antagonists that were examined include those such as proadifen

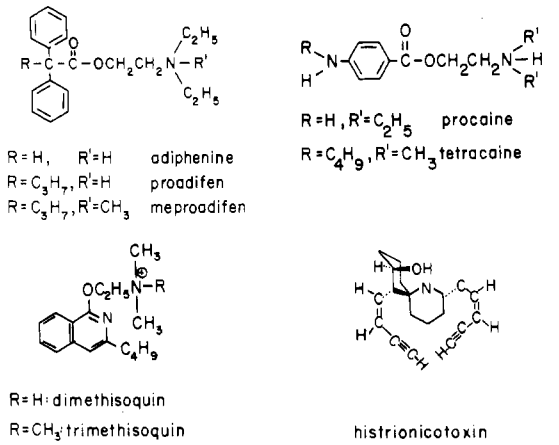

FIGURE 1: Structures of amine noncompetitive antagonists.

that enhance [^{3}H]AcCh equilibrium binding affinity as well as adiphenine, tetracaine, and perhydrohistrionicotoxin (H_{12}-HTX) that either had no effect or reduced [^{3}H]AcCh equilibrium binding affinity. Effects of these compounds individually on receptor conformational equilibria and in combination were defined by use of a [^{3}H]AcCh titration assay which provided an estimate of the fraction of receptors in the high-affinity conformational state. The results of these studies provide information about the number of different receptor conformations stabilized by amine noncompetitive antagonists and also about alternative mechanisms of receptor desensitization. A preliminary report of these studies has appeared (Cohen et al., 1980).

Materials and Methods

Preparation of Postsynaptic Membranes. Membranes were prepared from freshly dissected electric organs of *Torpedo californica* essentially as described by Sobel et al. (1977). The membranes were stored at 4 °C in 37% sucrose–0.02% NaN_3. Receptor site concentrations were determined by measuring the binding of [^{3}H]propionyl-α-bungarotoxin (Neubig & Cohen, 1979) or of [^{3}H]AcCh (Boyd & Cohen, 1980a). For 10 fish, the specific activity of the membrane suspensions was 1.6 ± 0.4 μmol of α-toxin sites/g of protein. Experiments were carried out at 4 °C with membrane suspensions in *Torpedo* physiological saline (TPS: 250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, $MgCl_2$, 5 mM NaP_i, and 0.02% NaN_3, pH 7.0) supplemented with 0.1 mM diisopropyl phosphorofluoridate to inhibit acetylcholinesterase activity.

Chemicals. Acetylcholine ([^{3}H]acetyl) was from Amersham (Arlington Heights, IL); [^{3}H]propionyl-α-bungarotoxin and $^{22}Na^+$ were obtained from New England Nuclear (Boston, MA). Nonradioactive cholinergic ligands and noncompetitive antagonists obtained commercially were used without further purification. α-Bungarotoxin (α-BgTx) was from Miami Serpentarium. Acetylcholine and carbamylcholine chloride as well as tetracaine hydrochloride were from Sigma Chemical (St. Louis, MO); adiphenine (diethylaminoethyl diphenylacetate) hydrochloride was from Pfaltz and Bauer (Stamford, CT). Hydrochloride salts of proadifen (diethylaminoethyl 2,2-diphenylpentanoate) and dimethisoquin were a gift from Smith, Klein and French (Philadelphia, PA). Meproadifen iodide was synthesized from proadifen as described (Krodel et al., 1979). Synthetic *dl*-perhydrohistrionicotoxin was a gift from Prof. Y. Kishi (Cambridge, MA). Structures of the noncompetitive antagonists are presented in Figure 1.

[^{3}H]AcCh Binding Assays. The binding of [^{3}H]AcCh to *Torpedo* membranes was determined by vacuum filtration on dry glass fiber filters (Whatman GF/F, 2.5 cm) as described

by Boyd & Cohen (1980a). To examine the effect of various noncompetitive antagonists on the equilibrium binding of [^{3}H]AcCh, membrane suspensions (40 nM α-BgTx sites, 36 nM [^{3}H]AcCh in TPS) were incubated for 30 min with the drug under examination, and a 5-mL aliquot of each incubation mixture was then filtered. The concentration of free [^{3}H]AcCh was determined by counting a 1-mL aliquot of the filtrate. [^{3}H]AcCh bound specifically to the *Torpedo* membranes was determined from the difference between the total radioactivity on the unwashed filter and the [^{3}H]AcCh retained at the same concentration of free [^{3}H]AcCh when membranes had been pretreated for 60 min with a 5–10-fold excess of α-BgTx to occupy all receptor sites. In this assay, more than 90% of the [^{3}H]AcCh retained on the filter is bound specifically to receptor, while about 3% results from adsorption of [^{3}H]AcCh to the filter itself. Separate determinations of the nonspecific binding were made for each concentration of added ligand, because aliphatic and aromatic amines (but not α-BgTx) reduced [^{3}H]AcCh binding to the glass filters.

The kinetics of binding of [^{3}H]AcCh for reaction times greater than 5 s were measured by manual mixing and filtration techniques. The specific binding of [^{3}H]AcCh to the membrane-bound receptor at the time of filtration (B'_t) was calculated from the total radioactivity retained on the filter and the free [^{3}H]AcCh in the filtrate by use of the same controls described for the equilibrium binding. The rate of dissociation of [^{3}H]AcCh–receptor complexes was measured as the rate of exchange of [^{3}H]AcCh for nonradioactive AcCh following addition of a 20-fold excess of AcCh.

[^{3}H]AcCh Titration Assay for Receptors in the High-Affinity Conformation. A specific experimental protocol based on known binding parameters of the high-affinity conformation was used to determine the fraction of AcCh binding sites existing in a conformation binding AcCh with high affinity (R_{Hi} of eq 1). In the absence of cholinergic ligands, ~15% of the AcCh binding sites exist in a conformation binding AcCh with high affinity (K_d = 2 nM) and with an association rate constant $k_+ = 3 \times 10^7$ M^{-1} s^{-1}, while the remaining sites bind AcCh with an affinity about 1000-fold lower (Boyd & Cohen, 1980a,b). Thus, when a membrane suspension containing 10 nM binding sites is incubated with 30 nM [^{3}H]-AcCh for 5 s, greater than 90% of the high-affinity sites but less than 3% of the low-affinity sites will be occupied. Furthermore, the short incubation time minimizes perturbations of the conformational equilibria due to the binding of [^{3}H]-AcCh itself since they occur at this concentration of [^{3}H]AcCh with a $T_{1/2}$ ~300 s. The simple, manual protocol used to achieve an incubation time of 5 s involved the rapid injection of a 4.5-mL volume of membrane suspension into a vortexed solution (0.5 mL) of 300 nM [^{3}H]AcCh contained in a 17 × 100 mm polypropylene test tube and termination of the binding reaction by filtration 5 s after mixing. The filter was rinsed rapidly (<2 s) with 8 mL of TPS to remove most of the [^{3}H]AcCh not bound specifically to the receptor. Since [^{3}H]AcCh bound to the high-affinity receptor conformation dissociates with $T_{1/2}$ ~ 18 s at 4 °C, this filter wash results in a relatively insignificant loss of [^{3}H]AcCh bound specifically to the high-affinity site. The specific binding determined in this manner divided by the total number of AcCh binding sites (determined by occupation of all sites following a 30-min incubation with 150 nM [^{3}H]AcCh) is equal to the fraction of receptors existing in a high-affinity conformational state.

The effect of noncompetitive antagonists on receptor conformational equilibria was examined following incubation for 30 min of a concentrated membrane suspension (2 μM α-BgTx binding sites, 0.1 mM DFP in TPS) with the desired concentration of drug. That suspension was then diluted 200-fold into TPS to reduce the drug concentration. At increasing time intervals following dilution, the fractions of AcCh binding sites remaining in a high-affinity conformation were measured by the titration assay described above. If the antagonist produces a reversible perturbation of the preexisting conformational equilibria, then following dilution the receptor will relax back to its unperturbed conformational state. The extent of perturbation and the kinetics of recovery are defined by this protocol. It should be emphasized that although the binding of [^{3}H]AcCh is used to measure the amount of high-affinity receptor, the initial perturbation by antagonist and the subsequent recovery occur in the absence of agonist. [^{3}H]AcCh was present only for the 5-s incubation necessary to define the amount of high-affinity binding.

Agonist-Induced $^{22}Na^+$ Flux. Suspensions of *Torpedo* postsynaptic membrane contain sealed vesicles, and the $^{22}Na^+$ efflux assay of Neubig & Cohen (1980) was used to measure the effects of noncompetitive antagonists on the agonist-induced permeability response. A concentrated membrane suspension (8 μM α-BgTx sites) was equilibrated overnight at 4 °C with 50 μCi of $^{22}Na^+$/mL, and conditions for unidirectional efflux were established by removal of the external $^{22}Na^+$ following column chromatography and dilution. $^{22}Na^+$ retention within vesicles was determined by filtration on glass fiber (Whatman GF/F) filters followed by a 10-mL wash. This assay was utilized to examine the effect on the functional state of the receptor of preincubation and then removal of noncompetitive antagonists. At the beginning of the efflux experiment, the concentrated membrane suspension was incubated with antagonist, and after the desired time, the antagonist concentration was reduced when the membrane suspension was passed over a 3-mL Dowex-50W ion-exchange column (Na^+ form) and then diluted 320-fold in TPS. At increasing time intervals following dilution, the responsiveness of the vesicles to agonist was measured by incubating an aliquot of the suspension with 0.3 mM phenyltrimethylammonium (PTA) for 20 s followed by filtration. The amount of $^{22}Na^+$ released by PTA was determined by the difference in radioactivity retained on the filters before and after the exposure to PTA. $^{22}Na^+$ retained nonspecifically was determined by measuring the radioactivity retained on the filters following treatment of the membrane suspension with the ionophore gramicidin (12.5 μg/mL). The use of the partial agonist PTA avoids the complication of spare receptors that exists in this assay for full agonists such as AcCh or carbamylcholine (Neubig & Cohen, 1980).

Results

Aromatic amine noncompetitive antagonists such as proadifen, dimethisoquin, and prilocaine are known to increase the affinity with which cholinergic agonists and competitive antagonists are bound at equilibrium by the membrane-bound *Torpedo* nicotinic receptor (Cohen et al., 1974; Krodel et al., 1979). This enhancement of receptor affinity is not, however, a general property of aromatic amine noncompetitive antagonists. Thus, when the equilibrium binding of [^{3}H]AcCh was determined for a concentration of [^{3}H]AcCh sufficient to occupy 60% of the receptor sites, proadifen from 1 to 100 μM produced a concentration-dependent increase in the [^{3}H]AcCh bound specifically to the receptor, while adiphenine, a close structural analogue of proadifen, did not increase the binding of [^{3}H]AcCh and even decreased slightly its equilibrium binding when present in the same concentration range (Figure 2). Tetracaine at 3 μM decreased [^{3}H]AcCh binding by 30%,

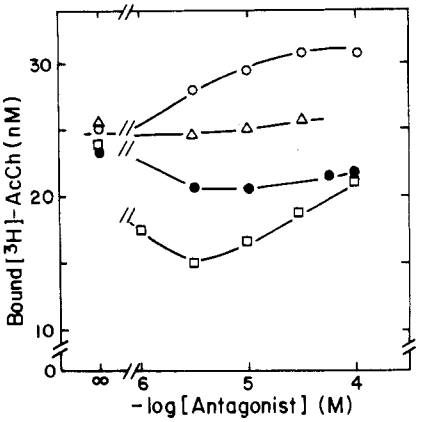

FIGURE 2: Effects of noncompetitive antagonists on the equilibrium binding of [^{3}H]AcCh to *Torpedo* receptor rich membranes. A membrane suspension (40 nM α-BgTx sites) was equilibrated with 36 nM [^{3}H]AcCh for 30 min at 4 °C in the presence of the indicated concentrations of proadifen (○), adiphenine (●), or histrionicotoxin (△). For tetracaine (□), suspensions were incubated for 3 h. [^{3}H]AcCh bound specifically to receptors was determined by ultrafiltration as described under Materials and Methods. Each point is the average of duplicate determinations.

while higher concentrations resulted in an increased binding of [^{3}H]AcCh, though the binding never exceeded that in the absence of drug (Figure 2). H_{12}-HTX at concentrations up to 30 μM produced a small effect, if any, on the equilibrium binding of [^{3}H]AcCh. The equilibrium dissociation constant (K_{eq}) for AcCh can be calculated from the known concentration of receptor sites and of bound and free [^{3}H]AcCh. The increased receptor occupancy in the presence of 100 μM proadifen results from a decrease in the K_{eq} from 7 to 2 nM, while the decreased binding caused by 3 μM tetracaine would be produced by an increase of K_{eq} to 35 nM.

Effects of Amine Noncompetitive Antagonists on the Receptor Conformational Equilibrium. We wished to determine whether the effects observed on the equilibrium binding of [^{3}H]AcCh were a consequence of alteration in the amount of receptor preexisting in the conformation binding AcCh with high affinity. To measure the amount of receptor binding [^{3}H]AcCh with high affinity (%R_{Hi}), the membrane suspensions were exposed to a low concentration of [^{3}H]AcCh (30 nM) for a brief time (see Materials and Methods). In Figure 3, a comparison is made of the effects of proadifen with that of the agonist carbamylcholine (Carb). When a membrane suspension was equilibrated with 3 μM Carb and then diluted 200-fold, the fraction of sites occupied by [^{3}H]AcCh in the assay decreased from 88% when determined 25 s after dilution to about 25% at 60 min. The latter value was higher than the 15% occupancy measured in membranes never exposed to Carb, and is expected since the Carb concentration following dilution is 15 nM and thus about 10% of the sites remain occupied, preventing full relaxation to the unoccupied value. When the membrane suspension was incubated with 20 and 60 μM proadifen, a similar, dose-dependent increase in high-affinity receptor was observed at short times after dilution, and this was followed by a slow recovery to about 15% R_{Hi} measured at 60-min postdilution. When the recovery kinetics were analyzed in terms of a semilogarithmic plot (Figure 3 inset), it was found that for both concentrations of proadifen, as for Carb, the recovery process was first order and the rate constants obtained from the slopes of the plots were similar and equal to $(2.2 \pm 0.2) \times 10^{-3}$ s^{-1}. Thus, with this experimental protocol, it was possible to determine for any concentration of drug both the amount of receptor sites converted to a conformation binding AcCh with high affinity and

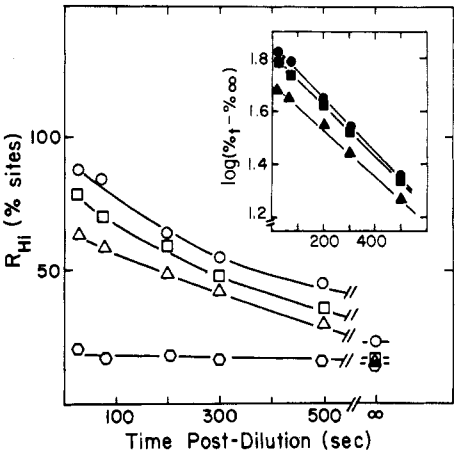

FIGURE 3: Stabilization of the high-affinity receptor conformation by proadifen: reversibility of conformational perturbation. *Torpedo* membrane suspensions (2 μM α-BgTx sites) were incubated with 3 μM Carb (○) or with 60 (□) or 20 μM (△) proadifen or were incubated in the absence of added drug (○). After a 30-min incubation at 4 °C, the suspension was diluted 200-fold in TPS. At increasing time intervals after dilution, the amount of high-affinity receptor (R_{Hi}) was determined by titration with [^{3}H]AcCh (30 nM, 5 s) as described under Materials and Methods. Inset: Semilogarithmic plot of the recovery kinetics after stabilization by Carb (●) or proadifen [20 (▲) or 60 μM (■)].

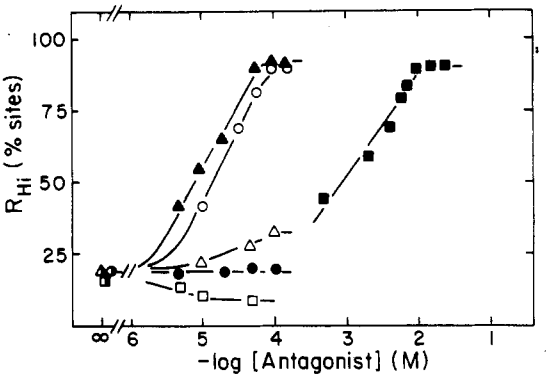

FIGURE 4: Concentration dependence of the stabilization of the high-affinity receptor conformation by amine noncompetitive antagonists. The percentage of cholinergic receptor binding sites in the high-affinity conformation was determined by a [^{3}H]AcCh titration assay described under Materials and Methods and in Figure 3. Concentrated membrane suspensions (2 μM α-BgTx sites) were preincubated for 30 min with proadifen (▲), dimethisoquin (○), lidocaine (■), H_{12}-HTX (△), adiphenine (●), and tetracaine (□). Stabilization of high-affinity receptor was determined after 200-fold dilution into physiological saline. Drug concentrations are the total concentration in the incubation mixture and not the free concentration.

the rate constant (k_{rec}) characterizing the reversal of that conformational shift after removal of the antagonist.

The capacity of several amine noncompetitive antagonists to stabilize the membrane-bound receptor in a conformation binding agonist with high affinity was measured with this protocol, and results are summarized in Figure 4. In the absence of added drug, 15–20% of the AcCh binding sites exist in a high-affinity conformation. Incubation of the membrane-bound receptor with proadifen, dimethisoquin, and lidocaine resulted in the dose-dependent stabilization of a high-affinity conformation. Exposure to high antagonist concentrations resulted in ~90% of the AcCh sites in a high-affinity conformation. Proadifen and dimethisoquin produced half-maximal effects when present at a total concentration (C_{50}) of about 10 μM, while lidocaine was characterized by a C_{50} of 1 mM. Adiphenine, on the other hand, produced no measurable increase in %R_{Hi} at concentrations

Table I: Conformational Perturbation of the *Torpedo* AcCh Receptor by Agonist and Noncompetitive Antagonists: Rate Constant (k_{rec}) for Receptor Reisomerization upon Removal of Antagonist[a]

membrane preparation	k_{rec} ($\times 10^3$ s^{-1})					
	carbamylcholine	proadifen	dimethisoquin	trimethisoquin	lidocaine	2-propanol
A	2.9 ± 0.4 (1)	3.1 ± 0.3 (6)	3.0 ± 0.3 (1)	3.2 ± 0.3 (2)		
B	2.4 ± 0.2 (3)	2.1 ± 0.2 (4)				2.3 ± 0.3 (4)
C	2.0 ± 0.2 (5)				2.0 ± 0.3 (2)	
D		2.1 ± 0.2 (1)	4.4 ± 0.2 (4)	3.0 ± 0.3 (3)	2.5 ± 0.3 (1)	2.4 ± 0.2 (2)

[a] Values of k_{rec} were determined as described under Materials and Methods and in Figure 3. Recovery kinetics were analyzed in terms of a semilogarithmic plot, and k_{rec} (±SD) was determined by a linear least-squares method. For each stabilizing ligand, the number of independent determinations (n) is indicated in parentheses; k_{rec} is an average value when $n > 1$.

as high as 0.1 mM. H_{12}-HTX produced a small increase in $\%R_{Hi}$ at concentrations above 10 μM, and tetracaine actually decreased the fraction of receptors binding AcCh with high affinity.

As for proadifen, after exposure to dimethisoquin, lidocaine, and the quaternary amine trimethisoquin, the kinetics of receptor reisomerization upon removal of antagonist were well characterized as a first-order process, and rate constants (k_{rec}) for several membrane preparations are summarized in Table I in comparison with values of k_{rec} for the agonist Carb. For these drugs, values of k_{rec} at 4 °C vary between 2×10^{-3} and 4×10^{-3} s^{-1}, and the variation from one drug to another was not greater than the variation from one membrane preparation to another. For any given membrane suspension, differences in k_{rec} exist; for example, k_{rec} for dimethisoquin was twice that for proadifen or lidocaine. Nevertheless, it is striking that the kinetics of recovery following stabilization and removal of the four noncompetitive antagonists were characterized by essentially the same rate constant as for the agonist Carb. For drugs such as adiphenine and H_{12}-HTX that produce only small perturbations, it was not possible to define k_{rec} directly, and further definition of their actions was obtained in studies of mixtures of antagonists (see below).

The effects of meproadifen were examined in experiments performed in parallel with proadifen. Meproadifen in a dose-dependent manner stabilized a high-affinity receptor conformation, but when the free concentration of meproadifen was reduced by dilution, there was only a very slow ($T_{1/2} \sim$ 50 min) decrease of the amount of receptor binding [^{3}H]AcCh with high affinity. This surprising result was observed in experiments on three different membrane suspensions, and the further analysis of the effects of meproadifen will be presented elsewhere.

The receptor conformation stabilized by proadifen was further characterized by the kinetics of dissociation of [^{3}H]-AcCh–receptor complexes as measured by the rate of exchange of [^{3}H]AcCh for nonradioactive AcCh in the presence and absence of proadifen (Figure 5). Following equilibration for 30 min with 150 nM [^{3}H]AcCh, the kinetics of dissociation of [^{3}H]AcCh–receptor complexes were characterized by a $T_{1/2} \sim$ 15 s, and the presence of 0.1 mM proadifen had no significant effect on the [^{3}H]AcCh dissociation kinetics. In the absence of proadifen, following incubation with 40 nM [^{3}H]AcCh for 10 s, about 18% of the sites were occupied, and [^{3}H]AcCh dissociated from those sites as a first-order process with the same rate constant. For membranes incubated with 0.1 mM proadifen for 30 min and then exposed to 40 nM [^{3}H]AcCh for 10 s, [^{3}H]AcCh was bound to greater than 90% of the sites, but the kinetics of dissociation of [^{3}H]AcCh from those sites were unmodified. In additional experiments, we established that the kinetics of dissociation of [^{3}H]AcCh at equilibrium were unmodified by the presence of dimethisoquin (10 μM) or adiphenine (60 μM).

Effects of Aliphatic Alcohols on the Receptor Conformational Equilibrium. The same [^{3}H]AcCh binding assay used to monitor the effects of amine noncompetitive antagonists was used to determine whether aliphatic alcohols also stabilized a receptor conformation binding [^{3}H]AcCh with high affinity. Membranes were incubated for 30 min with the desired alcohol concentration, the free alcohol concentration was reduced 200-fold by dilution, and at known times after dilution, the fraction of sites binding [^{3}H]AcCh with high affinity was determined by incubation with 30 nM [^{3}H]AcCh for 5 s. Irreversible effects of alcohols on the AcCh receptor were defined by the reduction in the number of sites occupied by 150 nM [^{3}H]AcCh. Incubation of the membrane suspensions with aliphatic alcohols (1-propanol, 2-propanol, 1-butanol, and *tert*-butyl alcohol) and with benzyl alcohol produced a dose-dependent increase in the amount of receptor in a high-affinity conformation (Figure 6). Half-maximal effects were observed for concentrations of 1- and 2-propanol of 2% (v/v). This concentration is similar to that for *tert*-butyl alcohol and slightly greater than that for 1-butanol (1%) of benzyl alcohol (~0.5%). For the more potent alcohols, this alteration of receptor conformational equilibria occurred at concentrations

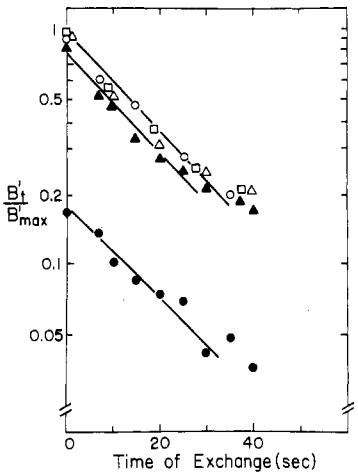

FIGURE 5: Kinetics of dissociation of [^{3}H]AcCh: effect of proadifen. The rate of dissociation of [^{3}H]AcCh was determined at 4 °C as the rate of exchange of [^{3}H]AcCh for nonradioactive AcCh. Equilibrium conditions were the following: A member suspension (20 nM α-BgTx sites) was equilibrated with 150 nM [^{3}H]AcCh without additional drug (○) or with 0.1 mM proadifen (△). At zero time, a small aliquot of nonradioactive AcCh was added with shaking to give a final concentration of 3 μM, and the specifically bound [^{3}H]AcCh, B'_t, was measured by filtration at increasing times following the addition. Preequilibrium (10-s incubation) conditions were as follows: A membrane suspension (40 nM α-BgTx sites) preincubated with 0.1 mM proadifen (▲) or without added drug (●) was mixed in a rapid-mixing system with an equal volume of a solution containing 80 nM [^{3}H]AcCh with (▲) or without (●) 0.1 mM proadifen. Bound [^{3}H]AcCh was determined at 10 s after mixing, and between 12 and 15 s, a small aliquot of concentrated nonradioactive AcCh was added to give a final concentration of 800 nM. The decrease in bound [^{3}H]AcCh (B'_t) following addition of the nonradioactive AcCh was measured by filtration at the indicated times.

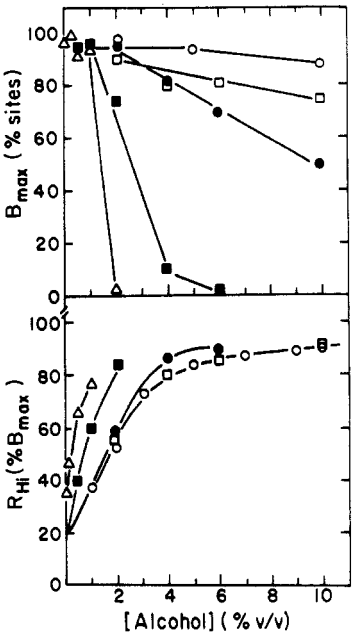

FIGURE 6: Effects of alcohols on membrane-bound AcCh receptor. Concentrated *Torpedo* membrane suspensions (2 μM α-BgTx sites) were incubated for 30 min at 4 °C with concentrations of 2-propanol (○), 1-propanol (●), *tert*-butyl alcohol (□), 1-butanol (■), or benzyl alcohol (△). Following a 200-fold dilution into *Torpedo* physiological saline, [^{3}H]AcCh binding properties were defined. (Upper panel) The number of sites (B_{max}) in each diluted suspension was determined by incubation for 20 min with 150 nM [^{3}H]AcCh and is expressed as a percentage of a control value in which no alcohol was added to the preincubation mixture. (Lower panel) The capacity of the alcohols in the absence of agonist to stabilize a high-affinity conformation was determined by a 5-s, 30 nM [^{3}H]AcCh titration assay (see Materials and Methods). Determinations were made at 15 and 35 s after dilution, and the percent at the time of dilution was estimated by extrapolation to zero time. The percentage of sites in a high-affinity conformation at the time of dilution is expressed relative to B_{max}.

only slightly lower than those causing irreversible loss of the normal equilibrium binding of [^{3}H]AcCh (Figure 6, upper panel). Benzyl alcohol and 1-butanol at concentrations of 1% (v/v) produced no decrease in the number of [^{3}H]AcCh binding sites, but concentrations of 2% and 4%, respectively, reduced the number of sites by more than 90%. The nature of this irreversible loss in high-affinity binding was not examined further. Further studies focused on 2-propanol since incubation with this alcohol at concentrations up to 5 times its C_{50} resulted in only a small decrease in the number of [^{3}H]AcCh binding sites.

Membrane-bound receptors were incubated with 2-propanol at concentrations of 3% and 10%, and the time course of recovery following removal of the alcohol by dilution was followed by using the experimental technique described under Materials and Methods and in Figure 3. The kinetics of receptor reisomerization were a first-order process, and the observed rate constant, $k_{rec} = (2.3 \pm 0.3) \times 10^{-3}$ s^{-1}, was the same as that determined in parallel for Carb and for proadifen (Table I). In addition, the dissociation rate constant for [^{3}H]AcCh complexes stabilized by 2-propanol was the same as that observed for similar complexes stabilized by proadifen (data not shown). Thus, the high-affinity receptor conformation stabilized by 2-propanol is indistinguishable by these criteria from that stabilized by proadifen or Carb.

Combinations of Noncompetitive Antagonists. The addition of H_{12}-HTX (Figure 7A) to incubation mixtures containing 2-propanol resulted in an increase in the fraction of receptors in a high-affinity conformation that was approximately equal to the sum of the independent effects. In sharp contrast to

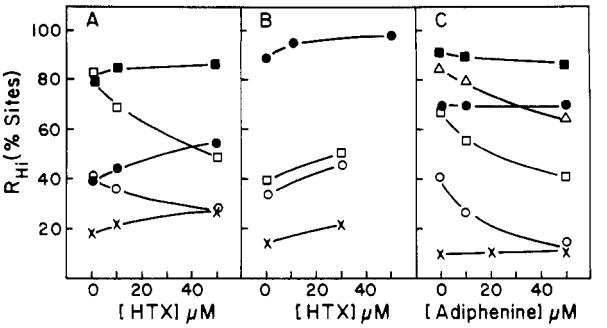

FIGURE 7: Perturbation of conformational equilibria of membrane-bound *Torpedo* receptor: effects of mixtures of noncompetitive antagonists. *Torpedo* membrane suspensions (2 μM α-BgTx sites) were incubated with the indicated drugs. After a 30-min incubation at 4 °C, the suspension was diluted into physiological saline, and the amount of high-affinity receptor was determined as described under Materials and Methods. (A) Membrane suspensions were incubated with H_{12}-HTX alone (×), with H_{12}-HTX and proadifen [5 μM (○), 60 μM (□)], or with H_{12}-HTX and 2-propanol [0.4 M (●), 1.3 M (■)]. (B) Membrane suspensions were incubated with H_{12}-HTX alone (×), with H_{12}-HTX and 3 μM Carb (●), or with H_{12}-HTX and *d*-tubocurarine [~0.4 μM (○), 5.6 μM (□)]. (C) Membrane suspensions were incubated with adiphenine alone (×), with adiphenine and proadifen [5 μM (○), 30 μM (□), 60 μM (△)], or with adiphenine and 2-propanol [0.5 M (●), 1.7 M (■)].

this observation, H_{12}-HTX antagonized in a dose-dependent manner the effects of proadifen alone. For example, in the absence of any added drug, the fraction (f) of binding sites in a high-affinity conformation was 0.2, and exposure of membrane suspensions to either 5 μM proadifen or 0.04 M 2-propanol increased that fraction to about 0.4. In the presence of 5 μM proadifen and 50 μM H_{12}-HTX, f was reduced to 0.25, the value observed for that concentration of H_{12}-HTX alone, while in the presence of 0.4 M 2-propanol and 50 μM H_{12}-HTX, f increased slightly to 0.5. While H_{12}-HTX antagonized the stabilization of high-affinity receptor by proadifen, H_{12}-HTX did not inhibit the stabilization of the high-affinity receptor conformation by the agonist Carb or the competitive antagonist tubocurarine (Figure 7B). H_{12}-HTX was not unique in its capacity to inhibit the effect of proadifen on receptor conformational equilibria, since adiphenine also acted in a dose-dependent manner to inhibit the effect of proadifen but not 2-propanol (Figure 7C).

Since neither H_{12}-HTX nor adiphenine on its own stabilizes a receptor conformation with high affinity for [^{3}H]AcCh, it was not possible to use a [^{3}H]AcCh titration assay to monitor conformational reequilibration after removal of these antagonists. However, it was possible to determine whether the presence of either H_{12}-HTX or adiphenine altered the kinetics of recovery observed for antagonists such as proadifen and propanol or for Carb. Although the presence of H_{12}-HTX or adiphenine reduced the fraction of receptors converted by proadifen to a high-affinity conformation (Figure 7), for all concentrations studied, the kinetics of receptor reisomerization following removal of antagonist followed first-order kinetics, and the values of k_{rec} were within experimental uncertainty (±15%) unmodified by the presence of adiphenine or H_{12}-HTX. Similarly, the presence of H_{12}-HTX or adiphenine did not modify the kinetics of recovery observed after removal of 2-propanol by dilution.

To probe further the conformational perturbation caused by adiphenine, we examined the effect of the presence of adiphenine during the recovery period following stabilization of the high-affinity receptor by the agonist Carb. A concentrated membrane suspension containing 2 μM α-BgTx sites was preincubated with 4 μM Carb and 80 μM adiphenine for

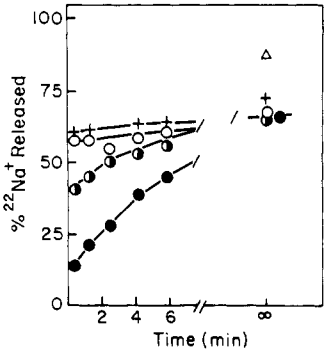

FIGURE 8: Agonist-induced $^{22}Na^+$ flux: noncompetitive antagonists. A *Torpedo* vesicle suspension (8 μM α-BgTx sites) was incubated overnight with $^{22}Na^+$ (50 μCi/mL). Conditions for unidirectional efflux were established by passing an aliquot over a 3-mL Dowex 50 cation-exchange column and diluting the suspension 300-fold in TPS. Before application to the column, suspensions were incubated for 60 s with 60 μM proadifen (●), 30 μM H_{12}-HTX (○), 30 μM H_{12}-HTX and 60 μM proadifen (◑), or no added drug (+). After dilution, aliquots (4.5 mL) were removed at the times indicated and added to a vortexed, 0.5-mL solution of 3 mM phenyltrimethylammonium, and the mixture was filtered 20 s later. Carbamylcholine [(△) 0.1 mM, 20 s] released 85% of the $^{22}Na^+$ for this vesicle preparation.

20 min. The membrane suspension was then diluted 200-fold in physiological saline containing 80 μM adiphenine, and the percentage of receptors binding [^{3}H]AcCh with high affinity was measured as shown in Figure 3. While recovery in the absence of adiphenine was characterized by a $T_{1/2}$ = 200 s, in the presence of 80 μM adiphenine there was a very rapid recovery with a $T_{1/2}$ < 15 s (data not shown). A similar protocol was used to monitor the effects of proadifen and tetracaine. When membrane suspensions were preincubated with Carb and 60 μM proadifen and then diluted into a solution containing 60 μM proadifen, there was no measurable decrease of the amount of high-affinity receptor, while for 75 μM tetracaine, the half-time for recovery was 200 ± 20 s, a value close to that measured in the absence of tetracaine (190 ± 20 s). Similar experiments were not conducted with H_{12}-HTX because of the limited quantities of toxin available. However, following preincubation with 3 μM Carb and 50 μM H_{12}-HTX, when the membrane suspension was diluted, the kinetics of recovery were the same as in the absence of H_{12}-HTX. That no observable recovery occurred in the presence of proadifen was consistent with the fact that proadifen on its own stabilizes a receptor conformation binding AcCh with high affinity. The rapid recovery observed in the presence of adiphenine suggests that the high-affinity conformation relaxes rapidly to a conformational state stabilized by adiphenine that does not bind [^{3}H]AcCh with high affinity and is different from the low-affinity state that exists in the absence of ligands.

Agonist-Stimulated $^{22}Na^+$ Fluxes: Desensitization by Noncompetitive Antagonists. The functional properties of receptor conformations stabilized by antagonists were examined by measuring the agonist-induced efflux of $^{22}Na^+$ from the *Torpedo* vesicles. An assay was utilized that defined the extent of desensitization caused by exposure to the antagonist in the absence of agonist and the rate of recovery from desensitization after removal of the antagonist. The effect of preincubation of membrane suspensions with 60 μM proadifen was compared with that of 30 μM H_{12}-HTX (Figure 8). Membrane suspensions containing $^{22}Na^+$ were incubated with each drug and also with a mixture of both for 60 s, and antagonist concentrations were then lowered by rapid column chromatography and dilution. The number of functional receptors was measured at increasing time intervals following removal of the antagonist by determining the amount of $^{22}Na^+$ released by the partial agonist phenyltrimethylammonium (PTA) (0.3 mM, 20 s). In the experiment shown in Figure 8, about 85% of the $^{22}Na^+$ was released from the vesicles when exposed to a maximal concentration of Carb. For membrane suspensions not incubated with antagonist, 0.1 mM PTA released 60–70% of the $^{22}Na^+$ in 20 s. Pretreatment with 60 μM proadifen caused a substantial reduction in the amount of $^{22}Na^+$ released by PTA, and at the first time point after 300-fold dilution of proadifen, only 12% of the $^{22}Na^+$ was released. The inhibition by proadifen was reversible, and within 30 min, the response to PTA was fully recovered to its unperturbed value. A semilogarithmic plot of the recovery kinetics yielded a straight line, indicating that the recovery was a first-order process with a $T_{1/2}$ of 250 s (data not shown). The value of the experimental rate constant, $k = 2.8 \times 10^{-3}$ s^{-1}, determined from the slope of the plot is within experimental error the same as the rate constant (k_{rec}, Table I) characterizing the reiosimerization of the high-affinity conformation to the low-affinity conformation upon removal of the stabilizing ligand. While 60 μM proadifen produced an 85% reduction in the PTA-induced $^{22}Na^+$ release as estimated by extrapolation to zero time, preincubation with 30 μM H_{12}-HTX for 60 s resulted in only a small (~5%) decrease in the percent of $^{22}Na^+$ released by PTA. Although preincubation with H_{12}-HTX alone was without effect on the subsequent response to PTA, H_{12}-HTX blocked desensitization by proadifen: when 30 μM H_{12}-HTX was added to the preincubation mixture of 60 μM proadifen, the inhibition was less than in the presence of proadifen alone. After incubation with 60 μM proadifen, the agonist-induced flux was blocked by 85%, but after incubation with 60 μM proadifen and 30 μM H_{12}-HTX, the response was only blocked by 30%. In both instances, the inhibition was fully reversible, and the recovery kinetics were characterized by the same rate constant.

In the previous experiments, membrane suspensions were preincubated with antagonist for only 60 s. In additional experiments, it was found that longer incubation with 60 μM proadifen produced no further inhibition and that the recovery kinetics following 60-s and 30-min preincubation were the same. For H_{12}-HTX, however, longer incubation produced complex effects. A 20-min preincubation with 40 μM H_{12}-HTX, a condition that did not result in significant stabilization of the high-affinity receptor, nevertheless resulted in greater than 80% inhibition of the PTA-induced $^{22}Na^+$ release; furthermore, recovery from this inhibition was very slow and characterized by a half-time of 15 min. Thus, while short preincubations with H_{12}-HTX can block the desensitization produced by proadifen, that effect does not represent an equilibrium effect of H_{12}-HTX, and a different mechanism for receptor inactivation is revealed after longer incubation times.

Preliminary experiments were conducted to determine whether preincubation with adiphenine or tetracaine altered the PTA-induced $^{22}Na^+$ flux. For adiphenine, a concentration of 40 μM was used since that concentration inhibited the conformational shift caused by 5 μM proadifen (Figure 7). Preincubation with 40 μM adiphenine and subsequent 300-fold dilution resulted in an initial inhibition by 50% of the PTA-induced $^{22}Na^+$ flux. Following dilution, the recovery of sensitivity to agonist was characterized by a $T_{1/2}$ ~ 160 s, i.e., a value similar to that observed with proadifen. For membrane suspensions preincubated with 20 μM tetracaine for either 60 s or 30 min and then diluted 300-fold, the PTA-induced $^{22}Na^+$ flux response was reduced only by about 10%. The inhibition

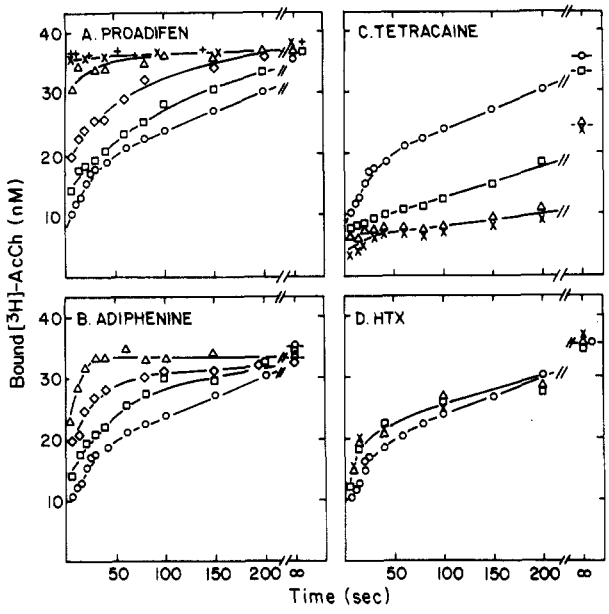

FIGURE 9: Effect of amine noncompetitive antagonists on the time course of [^{3}H]AcCh binding at 4 °C. Equal volumes of a suspension of *Torpedo* membranes incubated with drug and a solution of [^{3}H]AcCh and the same concentration of drug were mixed so that the final concentration in the reaction mixture was 37 nM α-BgTx sites and 106 nM [^{3}H]AcCh. Aliquots were filtered on GF/F filters at the indicated times after mixing. [^{3}H]AcCh specifically bound to receptor was calculated as described under Materials and Methods. Membrane suspensions were preincubated for 30 min with (A) 0 (○), 1 (□), 3 (◇), 10 (△), or 30 μM (×) proadifen. In one experiment (+), proadifen was omitted from the preincubation mixture, and the membranes were mixed with a [^{3}H]AcCh solution containing 60 μM proadifen. (B) Adiphenine: 0 (○), 3 (□), 10 (◇), or 120 μM (△). (C) Tetracaine: 0 (○), 1 (□), 10 (△), or 60 μM (×). (D) H_{12}-HTX: 0 (○), 1 (□), 3 (△), or 10 μM (×).

was reversible, but because of its small magnitude, it was not possible to define the half-time for recovery. These results indicate that the receptor conformation stabilized by adiphenine is a functionally desensitized conformation even though it is not the same conformation stabilized by agonist or proadifen. Tetracaine, on the other hand, does not appear to stabilize a desensitized receptor conformation.

Effects of Amine Noncompetitive Antagonists on the Kinetics of [^{3}H]AcCh Binding. Effects of noncompetitive antagonists on receptor conformations in the presence of agonist can be defined by an analysis of their effects on the kinetics of agonist binding. A full analysis requires data over a wide range of agonist and antagonist concentrations. However, the known kinetic parameters characterizing the binding of [^{3}H]AcCh in the absence of antagonist (Boyd & Cohen, 1980a) suggested that for a single concentration of [^{3}H]AcCh (100 nM), association kinetics would be quite sensitive to drug modifications of intrinsic receptor conformational rates and equilibria. We wished to determine whether adiphenine, H_{12}-HTX, and tetracaine had similar effects and to compare their effects with those of proadifen. Membrane suspensions were preincubated with noncompetitive antagonists for 30 min, and the treated suspension was mixed with an equal volume of buffer containing 200 nM [^{3}H]AcCh and the same concentration of drug. [^{3}H]AcCh bound specifically to the receptor (B'_t) was determined as a function of time after mixing by ultrafiltration (Figure 9). As expected, in the absence of noncompetitive antagonist, equilibrium was attained slowly, only many minutes after mixing. The half-time ($T_{1/2}$ = 70 s) for the slow component of the association process and the amount of binding associated with that process (B'_s, 76% of total binding) were determined from a plot of the logarithm of $B'_\infty - B'_t$ against time. For this concentration of AcCh, binding that occurs rapidly ($B'_f = B'_\infty - B'_s$) reflects primarily the rapid binding of [^{3}H]AcCh to the preexisting high-affinity receptor (K_d = 2 nM) with a small contribution from the low-affinity receptor conformation (K_d = 1 μM). The rate constant for the slow phase reflects the rate of conversion of AcCh–receptor complexes from the low- to the high-affinity conformation.

For proadifen (Figure 9A), pretreatment of the membranes with high concentrations (10 and 30 μM) resulted in most of the binding occurring rapidly with equilibrium attained less than 10 s after mixing. That rapid binding was not dependent upon preincubation with proadifen since the same rapid binding was measured when untreated membranes were mixed with a [^{3}H]AcCh solution containing 60 μM proadifen (i.e., final concentration 30 μM). At lower concentrations (1 and 3 μM), there was a dose-dependent increase in B'_f as well as a reduction in the $T_{1/2}$ for the conversion by AcCh of the remaining low-affinity receptors. The increase in B'_f was expected for a drug such as proadifen which on its own increases the amount of high-affinity receptors. Dimethisoquin, prilocaine, and lidocaine, which behave similarly to proadifen in stabilizing the high-affinity receptor state, also produced similar effects on the kinetics of [^{3}H]AcCh association (data not shown). The effects of H_{12}-HTX, adiphenine, and tetracaine on [^{3}H]AcCh association kinetics differ from that of proadifen and in fact are quite distinct in each case. H_{12}-HTX, which produced only a small increase in the fraction of high-affinity receptors, was found to cause only small modifications of the kinetic profile of [^{3}H]AcCh binding (Figure 9D). At 3 μM H_{12}-HTX, there was only a small increase in both B'_f and $T_{1/2}$ and there was no further modification in the presence of 10 μM H_{12}-HTX. In sharp contrast to the lack of a significant effect of H_{12}-HTX on the kinetics of [^{3}H]AcCh binding, adiphenine caused a clear decrease in the $T_{1/2}$ for the slow phase of the association kinetics as well as small increases in B'_f (Figure 9B). In the absence of any added drug, the rapid component of the binding resulted in occupancy of 26% of the sites, and the slow phase was characterized by $T_{1/2}$ = 80 s, in the presence of 10 and 120 μM adiphenine, B'_f was increased to 44% and 47% of the sites while $T_{1/2}$ was decreased to 25 and 10 s, respectively. In contrast, for 3 μM proadifen, B'_f was 49%, and $T_{1/2}$ was 40 s while at higher concentrations, as noted above, all [^{3}H]AcCh association occurred too rapidly to be measured.

The effects of tetracaine on [^{3}H]AcCh association kinetics (Figure 9C) differ dramatically from the other drugs studied. Preincubation with 1 μM tetracaine resulted in a decrease of B'_f from 26% to 17% of the sites as well as an increase in the half-time of the slow phase from 70 to 220 s. [^{3}H]AcCh association kinetics in the presence of 10 and 60 μM tetracaine were the same within experimental uncertainty: about 15% of the sites were occupied rapidly while the slow phase of association was further retarded and was characterized by $T_{1/2}$ = 480 s.

Discussion

Nicotinic noncompetitive antagonists block acetylcholine receptor function without preventing the binding of AcCh, and, thus, drugs of this general class provide potentially useful tools to analyze the mechanism of permeability control by AcCh receptors. In this study, we characterized the effects of amine and alcohol noncompetitive antagonists on receptor conformational equilibria. We wished to determine whether in the absence of agonist noncompetitive antagonists stabilize the same high-affinity receptor conformation as that stabilized by

agonist or whether noncompetitive antagonists stabilize novel receptor conformations. Direct measurement of [^{3}H]AcCh equilibrium binding provided a simple means to identify ligands that allosterically regulate AcCh binding affinity. Although proadifen enhanced AcCh binding affinity, other aromatic amines including adiphenine, a close structural analogue of proadifen, and tetracaine actually reduced equilibrium binding of [^{3}H]AcCh. However, the observed concentration dependence of the effects of adiphenine and tetracaine on the [^{3}H]AcCh equilibrium is not compatible with a competitive interaction at the AcCh binding site itself.

The differential effects of these noncompetitive antagonists on [^{3}H]AcCh equilibrium binding are correlated with the effects of cholinergic agonists on their binding to the allosteric site in *Torpedo* membranes. In studies using [^{3}H]H_{12}-HTX to monitor ligand binding to this site, proadifen, dimethisoquin, and lidocaine are all bound an order of magnitude more tightly in the presence of Carb than in its absence (Aronstam et al., 1981; Medynski & Cohen, 1980); adiphenine is bound with a similar affinity ($K \sim 7$ μM) in the presence or absence of agonist (Medynski, 1983), and for tetracaine, $K \sim 0.3$ μM in the absence of agonist while $K \sim 80$ μM in the presence of Carb (Blanchard et al., 1979). Direct measurement of [^{3}H]tetracaine binding indicates that this drug binds to the same site as the other noncompetitive antagonists (Strnad & Cohen, 1983). Subtle changes in structure of the amine noncompetitive antagonists change the nature of the linkage function between the AcCh and anesthetic site from positive to neutral or negative.

To characterize the receptor conformation stabilized by noncompetitive antagonists in the absence of agonist, we used a [^{3}H]AcCh titration assay based upon the kinetic parameters that characterize the binding of [^{3}H]AcCh to *Torpedo* postsynaptic membranes (Boyd & Cohen, 1980a,b; Neubig et al., 1982). Membranes were equilibrated with antagonist, and the antagonist concentration was then reduced by dilution. The fraction of receptor sites binding AcCh with high affinity at times after dilution was measured by incubating the membranes with low concentrations of [^{3}H]AcCh for a brief (5-s) interval. In agreement with the results of Heidmann & Changeux (1979b), and Young & Sigman (1981), we conclude that amine and alcohol noncompetitive antagonists that enhance [^{3}H]AcCh equilibrium binding affinity act in the absence of agonist to produce a dose-dependent increase in the amount of receptor in a conformation binding agonist with high affinity.

The stabilization of high-affinity receptor by amines such as proadifen and by alcohols (2-propanol) was reversible upon removal of antagonist by dilution, and the recovery occurred at a first-order process characterized by a rate constant ($k_{rec} \sim 0.003$ s^{-1}, $T_{1/2} \sim 200$ s) that was the same as that observed following stabilization by agonist (Table I). For any ligand for which the rate-limiting step for the recovery process was receptor reisomerization and not ligand dissociation from the receptor, the recovery rate constant should be characteristic of the receptor conformation stabilized by the drug. The similar values of k_{rec} indicate that both amine and alcohol antagonists stabilize the same high-affinity conformation as agonist. That the kinetics of dissociation [^{3}H]AcCh are unmodified by the presence of proadifen (Figure 6) or 2-propanol provides further support for this interpretation. The observed values of k_{rec} indicate that ligands such as proadifen and dimethisoquin must dissociate with a $T_{1/2} < 180$ s. No direct determination of dissociation kinetics has been made for these stabilizing antagonists. However, since proadifen binds to the allosteric site with $K \sim 1000$ nM, while [^{3}H]AcCh binds to its site with $K = 10$ nM, it would be reasonable to expect that proadifen dissociates more rapidly than [^{3}H]AcCh whose dissociation is characterized by $T_{1/2} = 15$ s. Recent results, however, indicate that for certain amine noncompetitive antagonists, the kinetics of dissociation are surprisingly slow. At 4 °C, the half-time of dissociation of [^{3}H]meproadifen from the anesthetic site is 30 min (Cohen et al., 1980); at 20 °C, the half-time of dissociation of [^{3}H]H_{12}-HTX is 60 min (Medynski, 1983), while for [^{3}H]phencyclidine it is 5 min (Eldefrawi et al., 1982; Oswald et al., 1983). In our experiments, direct determination of recovery kinetics following stabilization by H_{12}-HTX was not possible, but meproadifen was identified as an antagonist characterized by a very slow recovery time ($T_{12} \sim 50$ min).

Table II: Interaction of Noncompetitive Antagonists with *Torpedo* Membranes

	C_{50} (μM)[a]	allosteric site K_{eq} (μM)[b]	AcCh site K_p (μM)[c]
proadifen	10	3 ± 1	400 ± 100
dimethisoquin	15	18 ± 6	25 ± 8
trimethisoquin	15	35 ± 15	12 ± 6
lidocaine	1300	25000 ± 5000	1100 ± 200
dibucaine	30	60 ± 20	60 ± 6
H_{12}-HTX	20	5 ± 2	120 ± 20
adiphenine		5 ± 2	200 ± 70
tetracaine		0.3 ± 0.1	500 ± 100

[a] C_{50} is the total drug concentration producing half-maximal stabilization of the high-affinity receptor conformation. C_{50} is determined from the data of Figure 4 and similar experiments for trimethisoquin and dibucaine. To determine free concentrations, it would be necessary to correct for nonspecific partitioning. [b] Equilibrium binding (K_{eq}) at 4 °C as determined by displacement of [^{3}H]H_{12}-HTX. Data are from Medynski (1983). [c] K_p is the concentration reducing by 50% the initial rate of binding of ^{125}I-α-BgTx. Data are from Krodel et al. (1979) (proadifen, dimethisoquin, and trimethisoquin), Blanchard et al. (1979) (lidocaine, dibucaine, and tetracaine), and Heidmann et al. (1983) (H_{12}-HTX) and from unpublished observations (adiphenine).

Although amine and alcohol noncompetitive antagonists can stabilize the same high-affinity receptor conformation, they act by distinct mechanisms. Thus, the presence of adiphenine or H_{12}-HTX antagonized the stabilization of the high-affinity receptor conformation by proadifen but not by 2-propanol (Figure 7). The result is compatible with the notion that the amines regulate receptor conformational equilibria as a consequence of their binding to the allosteric site in the *Torpedo* acetylcholine receptor, while the alcohols act as nonspecific perturbants of the lipid structure. The antagonism observed for mixtures of proadifen and adiphenine is dramatic, and we have also established that adiphenine antagonizes the conformational perturbation produced by dibucaine (data not shown). Similar results, however, need not be observed for all other amine noncompetitive antagonists. Any particular compound can interact potentially with the AcCh binding site itself, and also it can partition into the lipid phase and act as a nonspecific membrane perturbant. For the aromatic amines stabilizing the high-affinity receptor conformation, the concentrations producing half-maximal conformational perturbation can be compared (Table II) to the binding constants characterizing their interactions with the allosteric site in the absence of agonist or with the AcCh site itself (as judged by the inhibition of the rate of binding of ^{125}I-α-BgTx). Proadifen binds 100-fold more tightly to the allosteric site than to the AcCh site. For dimethisoquin and trimethisoquin, however, the affinity for the allosteric site is similar to that for the AcCh site as judged by the inhibition of the rate of binding of ^{125}I-α-BgTx. Lidocaine inhibition of ^{125}I-α-BgTx binding is

characterized by $K_p \sim 1.1$ mM while in the absence of agonist it displaces [^{3}H]H_{12}-HTX with $K_{eq} \sim 25$ mM. This indicates that a compound such as lidocaine might stabilize the high-affinity receptor conformation as a result of binding to the AcCh site, while for dimethisoquin interactions with both the allosteric and AcCh sites might contribute. Heidmann et al. (1983) note that perturbations produced by lidocaine, trimethisoquin, and chlorpromazine are not blocked by H_{12}-HTX, but further work is required to determine whether the conformational perturbations do result from ligand interactions with the AcCh site.

The [^{3}H]AcCh titration assay permits a direct method to determine whether any antagonist acts on its own to stabilize receptor conformation with high affinity for AcCh. In the assay, adiphenine produced no change in the amount of high-affinity receptor while tetracaine actually decreased that amount. The assay does not, however, provide direct information about the conformation(s) stabilized by these ligands other than the fact that they bind AcCh sufficiently weakly ($K > 50$nM) as to be undetected. Several lines of evidence indicate that adiphenine and tetracaine actually cause significant and distinct modifications of receptor structure. (1) The Kinetics of association of [^{3}H]AcCh (100 nM) are enhanced in the presence of adiphenine and are qualitatively similar to those observed in the presence of proadifen, while the presence of tetracaine causes a dramatic reduction in the slow phase of the [^{3}H]AcCh association kinetics (Figure 9). (2) After preincubation with adiphenine and Carb, when recovery was measured in the presence of adiphenine, recovery occurred more than 1 order of magnitude more rapidly than that observed following stabilization by Carb or by proadifen alone. These complex actions of adiphenine and tetracaine differentiate these ligands from H_{12}-HTX, which on its own produced a small increase in the amount of high-affinity receptor without having any major effect on the kinetics of [^{3}H]AcCh association.

The effects of noncompetitive antagonists on the PTA-induced $^{22}Na^+$ flux from the *Torpedo* vesicles provide information complementary to that obtained in the binding studies. The receptor conformation stabilized by agonist has been shown to be functionally desensitized (Neubig et al., 1982), and in agreement with early studies of Sugiyama et al. (1976), preincubation with proadifen results in a reversible loss of responsiveness to agonist. The kinetics of recovery of responsiveness following reduction of proadifen concentration are the same as the decrease of the amount of high-affinity receptor. Preincubation with both H_{12}-HTX and proadifen produced less desensitization than for proadifen alone (Figure 8), a result that indicates that H_{12}-HTX can prevent the binding of proadifen without producing desensitization. As noted under Results, however, that capacity of H_{12}-HTX to block desensitization is not the consequence of the equilibrium interaction between H_{12}-HTX and the *Torpedo* membranes. Prolonged exposure to H_{12}-HTX resulted in a subsequent inhibition of the PTA-induced $^{22}Na^+$ flux that recovered only very slowly after removal of H_{12}-HTX by dilution. While adiphenine produced no measurable increase in the amount of high-affinity receptor conformation, adiphenine on its own resulted in a reversible inhibition of the agonist-induced flux. This observation provides additional evidence that adiphenine perturbs the preexisting conformational equilibria to convert the receptor to a nonresponsive state.

Collectively these results provide evidence for multiple pathways by which the nicotinic receptor can be converted to a nonfunctional state. By binding to a receptor allosteric regulatory site, proadifen and several other amine noncompetitive antagonists stabilize a high-affinity desensitized state which by the various criteria used in this report is identical with that stabilized by agonists at equilibrium. Alcohols such as 2-propanol also stabilize this same state but do so independently of the specific allosteric regulatory site. They might act by nonspecific partitioning into membrane lipid or by more limited interactions with the receptor–lipid interface (Heidmann et al., 1983). Adiphenine and H_{12}-HTX (after prolonged incubation) do not convert receptor to this high-affinity state but do stabilize a nonfunctional state. Finally, tetracaine does not appear to stabilize a desensitized state and in fact slows the AcCh-stimulated conversion to the high-affinity state. Further studies will be necessary to characterize the mechanism of noncompetitive antagonism by ligands such as adiphenine and tetracaine.

Registry No. AcCh, 51-84-3; H_{12}-HTX, 55254-30-3; Carb, 462-58-8; Na, 7440-23-5; proadifen, 302-33-0; dimethisoquin, 86-80-6; lidocaine, 137-58-6; adiphenine, 64-95-9; tetracaine, 94-24-6; trimethisoquin, 69311-91-7; meproadifen, 56538-56-8; benzyl alcohol, 100-51-6; *tert*-butyl alcohol, 75-65-0; dibucaine, 85-79-0; propanol, 71-23-8; 2-propanol, 67-63-0; butanol, 71-36-3.

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Spin-Labeling Study of Phosphatidylcholine–Cardiolipin Binary Mixtures†

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ABSTRACT: Electron paramagnetic resonance spectra of the spin-label probe 2,2,6,6-tetramethylpiperidinyl-1-oxy have been used to study the phase behavior of binary mixtures of different phosphatidylcholines (dipalmitoyl, distearoyl, and dioleoyl) with cardiolipin, using either calcium-free or calcium-containing cardiolipin (with a calcium:cardiolipin ratio of 1:2) samples. Results show that the nature of the fatty acid chains of the phosphatidylcholines (chain length and unsaturation) may influence the coexistence of different phases as well as does the nature of the cation linked to the cardiolipin.

Binary mixtures of lipids have been extensively investigated by many different techniques, with the aim of finding a correlation between composition and structure for these mixtures. One of the main goals has been to find whether different phases, and in particular immiscible fluid phases, may exist in the plane of a phospholipid bilayer. Such lipid phase separations are biologically significant: (i) they affect the lateral molecular motion and therefore are involved in immune processes (McConnell, 1978); (ii) by modifying the lateral compressibility of the membrane, they play an important role in the insertion of proteins (Shimshick & McConnell, 1973); and (iii) by changing the state of the lipidic environment around a protein, they modulate the enzymatic activity (Wisnieski et al., 1974). In addition, calcium-induced phase modifications can be involved in transport phenomena and fusion processes (Verkleij et al., 1979).

One example of the immiscibility of lipid phases is the dimyristoylphosphatidylcholine (DMPC)[1]–cholesterol mixture for which a great number of techniques or approaches have been used, including lateral diffusion coefficient measurements (Rubenstein et al., 1979), calorimetry (Melchior & Steim, 1979), freeze–fracture electron microscopy (Copeland & McConnell, 1980), and spin-labeling studies (Rubenstein et al., 1979; Recktenwald & McConnell, 1981). Several attempts have been made to discuss the properties of such binary mixtures in terms of phase diagrams (Shimshick & McConnell, 1973; Lee, 1977; Lentz et al., 1980).

A second example is the binary mixture of DMPC and cardiolipin. For this system, spin-labeling and lateral diffusion

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[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; CL, cardiolipin; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; EPR, electron paramagnetic resonance.

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