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Kinetics of Binding of [^{3}H]Acetylcholine and [^{3}H]Carbamoylcholine to *Torpedo* Postsynaptic Membranes: Slow Conformational Transitions of the Cholinergic Receptor†

Norman D. Boyd and Jonathan B. Cohen*

ABSTRACT: The kinetics of binding of [^{3}H]acetylcholine (AcCh) and [^{3}H]carbamoylcholine (Carb) to membrane-bound nicotinic receptor from *Torpedo* electric tissue have been measured on the second time scale by rapid mixing and ultrafiltration. The concentration dependence of the association kinetics of agonist binding and the kinetics of ligand dissociation and receptor reisomerization following the removal of agonist are analyzed in terms of a model in which the observed binding is by a single population of receptors that exists in the absence of agonist in two interconvertible conformations, one binding agonist weakly (R_1) and the other binding with high affinity (R_2). A computer simulation has been used to determine values of rate and equilibrium constants characterizing the ligand interactions with the two conformations and for the conformational equilibrium in the presence and absence of agonist. At 4 °C, R_1/R_2 = 4.5, and the half-time for isomerization for low to high affinity of unliganded receptor is equal to 200 s, while for receptors occupied by either AcCh or Carb the half-time is reduced to ~4 s. For AcCh the apparent dissociation constants of the low- and high-affinity conformations are 800 nM and 2 nM, respectively (K_{eq} = 8 nM), and for Carb the values are 30 μM and 25 nM (K_{eq} = 100 nM). The dissociation rate constant of [^{3}H]AcCh from R_2 is equal to 0.04 s^{-1}. The results are further discussed in terms of alternate less satisfactory reaction models and are compared with the receptor conformational equilibria deduced by the use of other kinetic techniques.

Permeability control by nicotinic cholinergic receptors is characterized by two kinetically distinct processes: the primary permeability response (channel opening) that occurs within a fraction of a millisecond of the release of acetylcholine (AcCh)[1] from nerve and the slow decrease of that permeability response (desensitization) that occurs when a constant concentration of AcCh or other agonist is maintained for seconds or longer. Electrophysiological data concerning both channel opening and desensitization have been interpreted in terms of models where it is postulated that the nicotinic receptor (the AcCh binding protein and its associated channel) exists in a limited number of conformations differing both in their ligand binding properties and in the functional state of the ion channel [for reviews, see Gage (1976), Magazanik & Vyskocil (1976), and Colquhoun (1979)]. Thus, an "open channel" conformation is transiently stabilized when AcCh or another agonist is bound by the receptor, while prolonged exposure to AcCh permits the formation of a thermodynamically preferred "desensitized" conformation.

To provide direct evidence for these postulated receptor conformations, it is necessary to characterize under identical conditions the conformational equilibria defined by ligand binding and the functional state of the ion channel. Nicotinic postsynaptic membranes isolated from *Torpedo* electric tissue provide a unique preparation for such studies [for a review, see Heidmann & Changeux (1978)]. The cholinergic receptor in those membranes remains functional since cholinergic agonists cause increased cation permeabilities (Popot et al., 1976) and exposure to agonists for minutes or longer results in desensitization (Sugiyama et al., 1976; Bernhardt & Neumann, 1978). The use of rapid-mixing and quenched-flow techniques permits quantitative characterization of agonist dose–response relations for the *Torpedo* vesicles as well as a definition of the kinetics of channel activation and desensitization (Neubig & Cohen, 1980).

Analysis of the kinetics of binding of cholinergic ligands has begun to provide a definition of the conformational equilibria of the cholinergic receptor. The effects of cholinergic ligands on the kinetics of binding of radiolabeled α-neurotoxins (Weber et al., 1975; Weiland et al., 1977; Barrantes, 1978; Quast et al., 1978; Weiland & Taylor, 1979) provided evidence for

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[1] Abbreviations used: AcCh, acetylcholine; α-BgTx, α-bungarotoxin; Carb, carbamoylcholine; DFP, diisopropyl phosphorofluoridate; TPS, *Torpedo* physiological saline (250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM sodium phosphate, pH 7.0, and 0.02% NaN_3).

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conformational transitions occurring on the second-minute time scale, although the results have been interpreted in terms of different reaction mechanisms. An alternate approach capable of millisecond temporal resolution is the use of stopped-flow fluorescence techniques in conjunction with an appropriate signal. The fluorescence of the *Torpedo* membrane proteins provides a direct signal, since the binding of cholinergic agonists results in quenched tryptophan fluorescence (Bonner et al., 1976; Barrantes, 1978). Analysis of that signal has been limited by its small (≤1%) relative magnitude. Environmentally sensitive extrinsic fluorescent probes that have been introduced include quinacrine, a noncompetitive antagonist (Grunhagen et al., 1977), ethidium (Quast et al., 1979), and a cholinergic agonist (Heidmann & Changeux, 1979a,b). Interpretation of results obtained with the extrinsic probes may be complicated by the fact that they modify the nicotinic response. Measurement of the kinetics of binding of AcCh would provide a direct determination of receptor conformational equilibria and would provide useful data for direct comparison with the results of functional studies.

We report an analysis of the kinetics of binding of [^{3}H]-AcCh and of the agonist [^{3}H]carbamoylcholine (Carb) to the *Torpedo* receptor-rich membranes. A manual filtration technique was used to measure ligand binding at times in excess of 2 s after mixing membrane suspensions with agonist. The observed concentration dependence of the kinetics of ligand association and the kinetics of ligand dissociation and of receptor reisomerization after removal of cholinergic agonist are well accounted for by a reaction scheme consisting of a single binding site that exists in two conformations, each binding AcCh with different affinities. In the accompanying paper (Boyd & Cohen, 1980), we describe a further analysis of agonist binding kinetics obtained by the use of an automated rapid-mixing ultrafiltration apparatus possessing a temporal resolution of 0.2 s. Preliminary reports of these studies have appeared (Cohen & Boyd, 1977, 1979; Boyd & Cohen, 1978).

Materials and Methods

Preparation of Torpedo Postsynaptic Membranes. Receptor-enriched membranes were isolated from fresh *Torpedo marmorata* electric tissue as described (Krodel et al., 1979), while those isolated from the larger fish, *Torpedo nobiliana* and *Torpedo californica*, were isolated according to the procedure of Sobel et al. (1977). The binding of α-[^{3}H]neurotoxin from *Naja nigricollis* was used to estimate the concentration of cholinergic receptor sites (Weber & Changeux, 1974; Neubig & Cohen, 1979). Proteins were assayed by the method of Lowry et al. (1951) with bovine serum serum albumin as a standard.

Radiochemicals. Stock solutions of [^{3}H]acetylcholine chloride were stored at −20 °C in ethanol at a concentration of 10 mM. Radiochemical purity of [*acetyl*-^{3}H]AcCh (Amersham) was established by thin-layer chromatography (Lewis & Eldefrawi, 1974) and was at all times >95%. That chromatographic system was utilized to repurify [^{3}H]AcCh before use for certain experiments and also to determine the extent of hydrolysis of [^{3}H]AcCh during the course of ligand binding experiments. Isotope dilution experiments using the cholinergic receptor as binding protein (Neubig & Cohen, 1979) were utilized to determine specific activities of the [^{3}H]AcCh. Observed specific activities of the lots used were 500, 250, and 220 mCi/mmol, while the nominal specific activities supplied by the manufacturer were 147, 250, and 250 mCi/mmol, respectively. For experiments requiring [^{3}H]AcCh of higher specific activity, acetyl[*N-methyl*-^{3}H]choline obtained on an experimental basis from New England Nuclear was used. Radiochemical purity was determined by electrophoresis (3 mA, 30 min) on plastic-backed cellulose plates (Eastman 6064, 5 × 20 cm) in 2% formic acid–8% acetic acid pH 1.9 buffer. Under these conditions the mobility of [^{3}H]choline was 12.0 cm while that of [^{3}H]AcCh was 10.7 cm, and the procedure was used to repurify the ligand as necessary. Isotope dilution experiments established specific activities of 12 and 30 Ci/mmol for the batches used. Carbamoyl[*N-methyl*-^{3}H]choline was synthesized from [*N-methyl*-^{3}H]choline (New England Nuclear, 1 Ci/mmol) according to published procedures (Major & Bonnett, 1945). The same electrophoresis system was used to separate [^{3}H]choline from [^{3}H]Carb as was used for acetyl[*N-methyl*-^{3}H]choline.

Equilibrium Binding of [^{3}H]AcCh and [^{3}H]Carb. The binding of [^{3}H]AcCh and [^{3}H]Carb to membrane suspensions in *Torpedo* physiological saline (TPS, containing 250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM sodium phosphate, pH 7.0, and 0.02% NaN_3) was determined by vacuum filtration through glass fiber filters (Neubig et al., 1979). For experiments with [^{3}H]AcCh, membrane suspensions were pretreated with diisopropyl phosphorofluoridate (DFP) to inhibit acetylcholinesterase, and binding was determined in the presence of 0.1 mM DFP. A concentrated membrane suspension in TPS was incubated 60 min at 4 °C with 1 mM DFP, and the membranes were then diluted to the desired receptor concentration before the addition of [^{3}H]-AcCh. Under these conditions, acetylcholinesterase was inhibited by 99.9%, and the residual hydrolysis of AcCh was 1% per h for typical membrane preparations.

The ultrafiltration assay was designed to determine ligand binding to the membrane-bound receptor from the radioactivity retained on the filter. Since we wished to measure binding of [^{3}H]AcCh that might be rapidly reversible, binding was determined *without* washing the filters to remove free ligand. When a membrane suspension equilibrated with [^{3}H]AcCh is filtered, the [^{3}H]AcCh retained in the filter includes not only AcCh bound to the receptor but also [^{3}H]AcCh free in the fluid retained within the filter and [^{3}H]AcCh bound nonspecifically to the filter or to the membranes (see Results). Since the presence of α-bungarotoxin (α-BgTx) had no effect on the [^{3}H]AcCh retained by filters when solutions containing no membranes were filtered, [^{3}H]AcCh bound specifically to the receptor was determined from the difference between the total [^{3}H]AcCh retained on the filter and the [^{3}H]AcCh retained when membranes pretreated with excess α-BgTx to occupy all receptor sites were incubated with the same free [^{3}H]AcCh.

The precision of the assay depends upon the reproducibility of the fluid retention within the filter, and the following protocol provided most satisfactory results. A vacuum filtration apparatus (Hoeffer, San Francisco, CA) that held ten filters, with each filter well controlled by individual stopcocks so that each filtration occurred at an identical vacuum, was used. Filters were held in place by Teflon-coated filter weights. An aliquot of a membrane suspension equilibrated with [^{3}H]AcCh was pipetted onto a dry filter (Whatman GF/F), and then its stopcock was opened to the previously evacuated filtration chamber (27-in. Hg vacuum). The stopcock was left open for 5 s, permitting partial drying of the filter. Trivial sources of variability that have been eliminated include the droplets that fall from the filter weight onto the filter (hence the Teflon-coated weights) and variations of filtration pressure that alter the fluid retention of the filter. Dried filters were counted in a toluene scintillation cocktail (3 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]-

benzene/L). Total and free [^{3}H]AcCh were determined by counting aliquots (0.5 mL) of the total reaction mixture and the filtrate, respectively.

Kinetics of Binding of [^{3}H]AcCh and [^{3}H]Carb. The ultrafiltration assay was combined with a simple rapid mixing system to permit analysis of the kinetics of ligand binding at times greater than 1 s after mixing drug and receptor. The mixing system consisted of two syringes mounted in a Lucite block and connected by Teflon tubing to a "T" mixer (Hamilton No. 86516, 1.5-mm inner diameter). Mixing was achieved by forcing the syringe contents through the mixing chamber. At the pressures used, the velocity of fluid flow was determined to vary between 320 and 850 cm/s. Under these conditions, >95%, mixing is expected within 10 ms (Roughton & Chance, 1963). The efficiency of mixing was also determined visually by mixing solutions of different pH in the presence of phenol red.

When the kinetics of ligand association were to be determined, one syringe contained *Torpedo* membranes in TPS preincubated with DFP and the second syringe contained the desired concentration of [^{3}H]AcCh or [^{3}H]Carb in TPS. The ligand binding at time intervals between 1 and 15 s after mixing was determined by injecting the reaction mixture directly into the well of the filtration apparatus, where it was allowed to age for the desired interval until the time of filtration. In this manner the time of filtration can be defined within several tenths of a second. Association kinetics at times greater than 10 s after mixing were determined by collecting a large volume of the reaction mixture and then filtering aliquots at the desired time (Neubig et al., 1979). [^{3}H]AcCh bound specifically to the membrane-bound receptor at each time (B_t') was calculated from the total radioactivity retained on the filter by using the same controls described for equilibrium binding.

The rate of dissociation of [^{3}H]AcCh from the receptor-rich membranes at equilibrium can also be measured by this technique. In this case one syringe contained membranes equilibrated with the desired concentration of [^{3}H]AcCh, and the second syringe contained a high concentration of nonradioactive AcCh in TPS.

Results

Equilibrium Binding of [^{3}H]AcCh. In Figure 1 is shown the result of a typical experiment in which the equilibrium binding of [^{3}H]AcCh by *Torpedo* postsynaptic membranes was determined by ultrafiltration on glass fiber filters. When a membrane suspension equilibrated with [^{3}H]AcCh concentrations between 10 and 800 nM was filtered, the [^{3}H]AcCh retained on the filter was characterized by both a saturable, high-affinity component and a linear component. The linear component was equal to the [^{3}H]AcCh retained by filters when solutions containing no membranes or membrane suspensions pretreated with α-BgTx were filtered. Included in Figure 1 (inset) is a Scatchard plot of the specifically bound [^{3}H]AcCh containing data pooled from three experiments. For those experiments, the Scatchard plot was linear and the equilibrium binding of [^{3}H]AcCh at 4 °C was characterized by a dissociation constant, K_{eq} = 8 ± 1 nM. The concentration of [^{3}H]AcCh binding sites was equal to 1.1 ± 0.2 times the concentration of sites for the α-[^{3}H]toxin of *N. nigricollis* [a detailed discussion of site stoichiometries has been presented elsewhere (Neubig & Cohen, 1979)].

Kinetics of Ligand Binding: Ultrafiltration Assay. The use of the ultrafiltration assay to measure the kinetics of binding of [^{3}H]AcCh is shown in Figure 2. The total [^{3}H]AcCh retained on filters and the free [^{3}H]AcCh in the filtrate are

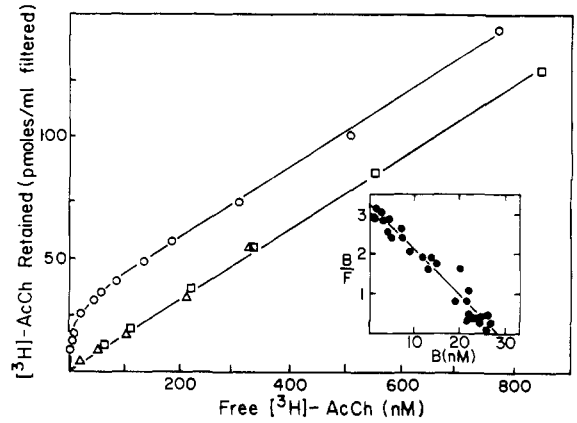


FIGURE 1: Equilibrium binding of [^{3}H]AcCh to *T. californica* membranes determined by ultrafiltration. A membrane suspension (25 nM α-toxin sites in TPS and 0.1 mM DFP) was equilibrated with concentrations of [^{3}H]AcCh for 30 min at 4 °C. 1-mL aliquots were then filtered on 2.5 cm Whatman GF/F filters as described under Materials and Methods. (○) Total [^{3}H]AcCh retained on the filters as a function of free (filtrate) [^{3}H]AcCh. (□) [^{3}H]AcCh retained when the membrane suspension was pretreated with α-BgTx prior to the addition of [^{3}H]AcCh. (△) [^{3}H]AcCh retained on the filters when 1-mL aliquots of [^{3}H]AcCh in TPS (no membranes) were filtered. (Inset) Scatchard plot of specific [^{3}H]AcCh binding to *T. californica* membranes determined from the difference between total and nonspecific retention of AcCh by the filters. Pooled data from three independent experiments.

plotted (Figure 2A) as a function of the reaction time (the time between mixing and filtration) for a membrane suspension containing 25 nM α-toxin sites and 100 nM [^{3}H]AcCh. A continuous increase of the [^{3}H]AcCh retention was observed up to ~100 s, after which no further increase was observed up to 30 min. [^{3}H]AcCh bound specifically to the receptor at each time (Figure 2B) was determined from the [^{3}H]AcCh retained in the filter by subtracting the nonspecific component of the retained [^{3}H]AcCh characteristic of that concentration of free [^{3}H]AcCh. That nonspecific component varies as a function of time because the free [^{3}H]AcCh varies with time. The complexity of the [^{3}H]AcCh association kinetics is seen when the data are replotted as a semilogarithmic plot (inset of Figure 2B). For this experiment, 25% of sites were occupied within 1 s, 25% of the reaction was characterized by a $t_{1/2}$ of ~3 s, and the remainder was characterized by a half-time, $t_{1/2}$ = 16 s. In this report, the association kinetics characteristic of the slowest phase of the ligand binding reaction are analyzed.

Kinetics of Binding of [^{3}H]AcCh and [^{3}H]Carb to Membrane-Bound Cholinergic Receptor of Torpedo. In Figure 3 are presented examples of the slow binding kinetics determined at 4 °C for a single membrane preparation at [^{3}H]AcCh concentrations below 100 nM (Figure 3A) and at [^{3}H]AcCh concentrations up to 660 nM (Figure 3B). The specific binding at each time (B_t') was determined from the [^{3}H]AcCh retained on the filters, and that binding was plotted as log ($B_\infty' - B_t'$) as a function of time. For all AcCh concentrations, the slow phase of the association kinetics was found to be linear in such plots, indicating a first-order process and permitting the determination of the rate constant (k_s) and the amplitude (B_s') of the slow phase from the slope and intercept, respectively. In addition, the amount of binding occurring rapidly (B_f') is equal to $B_\infty' - B_s'$. At the lower concentrations of AcCh where there were significant changes of free [^{3}H]AcCh (F) during the binding reaction, plots of log ($F_t - F_\infty$) as a function of time were also constructed. From the slope of these plots, the rate constant characterizing the decrease in free AcCh was obtained and shown to be, as expected, identical with that

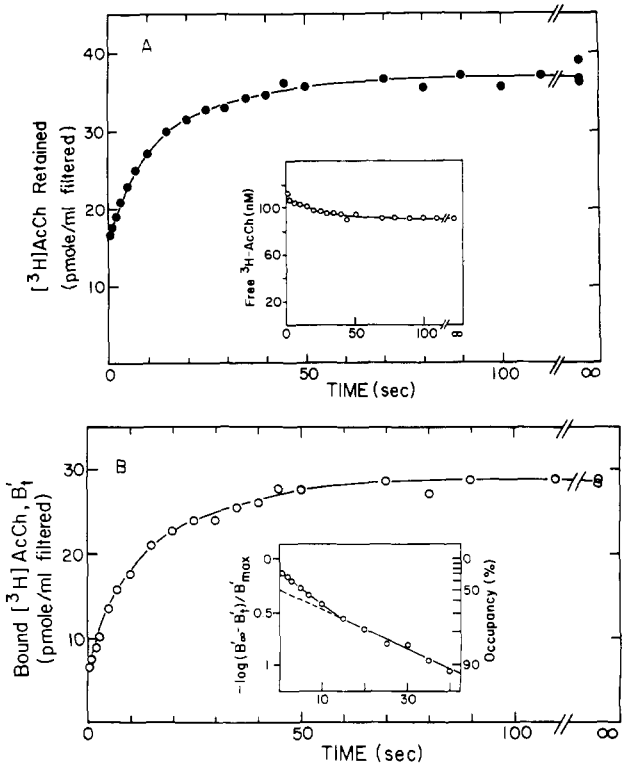


FIGURE 2: Kinetics of binding of [^{3}H]AcCh to *T. marmorata* membranes determined by ultrafiltration. Equal volumes of a *Torpedo* membrane suspension (50 nM α-toxin sites in TPS) and a solution containing 200 nM [^{3}H]AcCh in TPS (23 °C) were mixed, and 1.7-mL aliquots were filtered as described under Materials and Methods. (A) Total [^{3}H]AcCh retained per filter (●) when aliquots were filtered at the indicated times after mixing. (Inset) Free [^{3}H]AcCh (○). (B) [^{3}H]AcCh bound specifically to *Torpedo* receptor. This was calculated from the data of (A) and an appropriate control experiment utilizing membranes pretreated with α-BgTx to block all receptor sites. (Inset) Semilogarithmic plot of the extent of binding. B_∞' is the specific binding of [^{3}H]AcCh at equilibrium; B_{max}' is the concentration of AcCh binding sites.

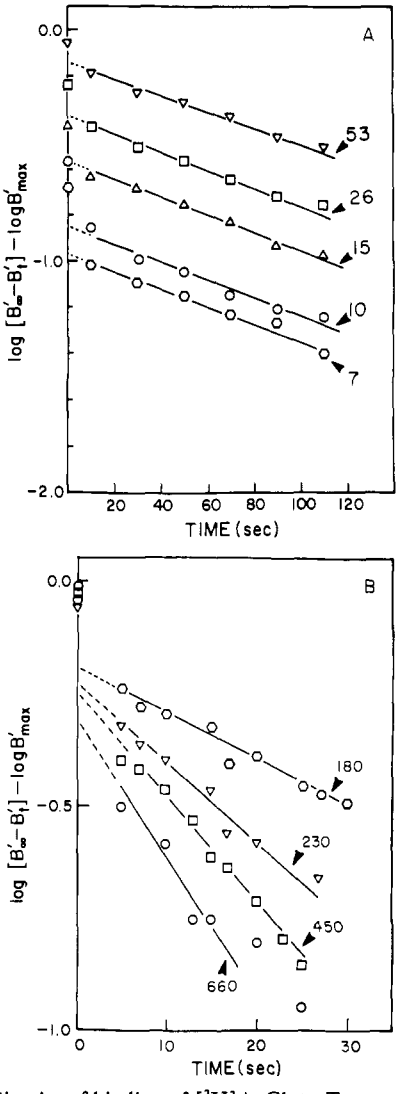


FIGURE 3: Kinetics of binding of [^{3}H]AcCh to *T. marmorata* membranes: dependence of the slow component on AcCh concentration. Equal volumes of a suspension of receptor-rich membranes and solutions containing [^{3}H]AcCh in TPS (4 °C) were mixed, and aliquots were filtered on 1.27-cm filters at the indicated times. The specific binding of [^{3}H]AcCh at each time (B_f') and at equilibrium (B_∞') was determined from the [^{3}H]AcCh retained on the filters as described under Materials and Methods. The reaction kinetics for each concentration are normalized in terms of B_{max}', the concentration of [^{3}H]AcCh binding sites. In this manner, the indicated ordinate values at zero time for each [^{3}H]AcCh concentration are log (B_∞'/B_{max}'), i.e., the logarithm of the fractional receptor occupancy at equilibrium for that concentration of [^{3}H]AcCh. The observed slow phase of the ligand association is analyzed in terms of reaction rate constants and amplitudes as follows. For each concentration of [^{3}H]AcCh, the slope of the semilogarithmic plot determines the rate constant (k_s) for the slow phase of the association reaction. Extrapolation of the observed binding to zero time determines the amount of binding occurring during the slow phase (B_s') and thus the amplitude of the binding occurring rapidly, $B_f' = B_\infty' - B_s'$. (A) The final reaction mixture was 25 nM in α-toxin sites and 7 (○), 10 (○), 15 (△), 26 (□), or 53 nM (▽) in [^{3}H]AcCh. (B) The final reaction mixture was 80 nm in α-toxin sites and 180 (○), 230 (▽), 450 (□), or 660 nM (○) in [^{3}H]AcCh.

characterizing the corresponding increase in bound AcCh. F_0, the concentration of free [^{3}H]AcCh calculated from the zero time intercept, was taken as a measure of the free [^{3}H]AcCh at the end of the rapid phases of the AcCh binding reaction. For comparison purposes, similar experiments were conducted also with [^{3}H]Carb.

The dependence of k_s upon agonist concentration is shown in Figure 4. The limiting value of k_s for both AcCh and Carb at the lowest concentration studied was equal to 0.0035 s^{-1}. k_s was approximately independent of AcCh concentration below 50 nM, and as the AcCh concentration increased from 50 nM to 500 nM, k_s increased an order of magnitude from 0.004 to 0.04 s^{-1}. This insensitivity of the k_s at low concentrations to changes in the AcCh concentration accounts for the absence of deviations from the observed first-order kinetics (Figure 3A) since under our experimental conditions, the concentration of free AcCh changes significantly over the time course of the reaction only in this same low concentration range. At a fixed concentration of free AcCh (F_0), k_s varied <20% as the concentration of receptor sites was varied 20-fold from 5 to 100 nM. For Carb, no increase of k_s was observed until concentrations above 500 nM, and k_s actually decreased from 0.0035 ± 0.0003 s^{-1} at 10 nM to 0.0025 ± 0.0002 s^{-1} at 200 nM. This small decrease is significant in terms of the models that can account for the data (see Discussion).

The variation of B_f' with ligand concentration (F_0) is presented in Figure 5 in comparison with the observed equilibrium binding (B_{eq}') as a function of free ligand concentration at equilibrium (F_{eq}). For each ligand, the equilibrium binding is well-defined by single population of binding sites characterized by K_{eq} = 8 nM for [^{3}H]AcCh and K_{eq} = 120 nM for [^{3}H]Carb. In contrast, the concentration dependence of B_f' must be characterized formally by two dissociation constants, K_H and K_L. The same fraction of sites (20–22%) bound both ligands initially with high affinity, characterized by K_H of 1.4 nM and 25 nM for [^{3}H]AcCh and [^{3}H]Carb, respectively. For

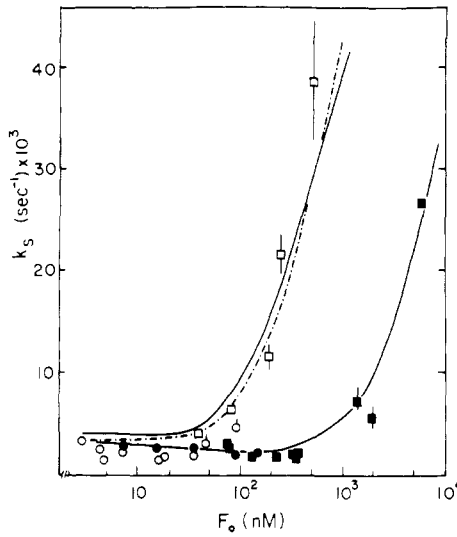


FIGURE 4: Kinetics of binding of [^{3}H]AcCh and [^{3}H]Carb to *T. californica* membranes (4 °C): dependence of k_s on agonist concentration. For each agonist concentration, the rate constant (k_s) characterizing the slowest phase of the binding reaction was determined from a semilogarithmic plot of the observed association kinetics (as in Figure 3). (Abscissa) F_0 is the free agonist concentration at the end of the rapid phases of the binding. For [^{3}H]AcCh, the concentration of α-toxin binding sites was 5 (○) or 100 nM (□); for [^{3}H]Carb the site concentration was 50 (●) or 100 nM (■). Solid curves were calculated according to eq 1 and the parameters in Table I. The broken curve for AcCh was calculated according to the parameters of Table I except K_{D1} = 1 μM and k_2' = 0.09 s^{-1}.

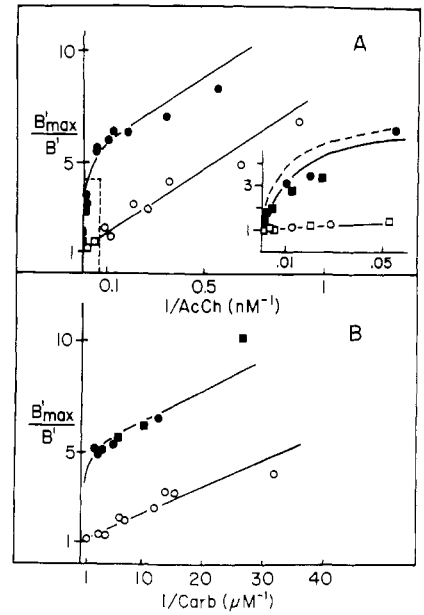


FIGURE 5: Kinetics of binding of [^{3}H]AcCh and [^{3}H]Carb to *T. calfornica* membranes (4 °C): dependence of reaction amplitude (B_f') on agonist concentration. For each concentration of [^{3}H]AcCh (A) or [^{3}H]Carb (B), B_f', the binding occurring during the rapid association phases, and F_0, the free agonist concentration associated with that amplitude, were determined from the time-dependent agonist binding during the slow phase (as in Figure 3). The concentration of binding sites in the reaction mixture was either 5 (●) or 100 nM (■) for experiments with [^{3}H]AcCh and 50 (●) or 100 nM (■) for the experiments with [^{3}H]Carb. (○) Binding of [^{3}H]agonist at equilibrium determined simultaneously with the association kinetics. Solid curves for AcCh and Carb were calculated according to the parameters of Table I. The broken curve for [^{3}H]AcCh was calculated according to the parameters of Table I except K_{D1} = 1 μM and k_2' = 0.09 s^{-1}. (Inset) Expanded presentation of the data at [^{3}H]AcCh concentrations above 25 nM.

[^{3}H]AcCh the low-affinity component of B_f' was characterized by K_L equal to 1000 nM, while a similar determination was not made for [^{3}H]Carb.

Kinetics of Dissociation of [^{3}H]AcCh Receptor Complexes. To further characterize the ligand binding properties of the *Torpedo* receptor, we measured the kinetics of dissociation of [^{3}H]AcCh–receptor complexes under preequilibrium as well as equilibrium conditions. In particular we wished to determine (1) whether the dissociation of [^{3}H]AcCh–receptor complexes at equilibrium was characterized by a single dissociation rate constant and (2) whether the dissociation of [^{3}H]AcCh–receptor complexes formed during the initial, rapid, high-affinity binding was characterized by similar kinetics.

The rate dissociation of [^{3}H]AcCh–receptor complexes at equilibrium was measured by the rate of exchange of [^{3}H]-AcCh for nonradioactive AcCh after a membrane suspension equilibrated with [^{3}H]AcCh was mixed with an equal volume of a solution containing excess nonradioactive AcCh. In addition, the known concentration dependence of the [^{3}H]AcCh association kinetics was used to design an experimental protocol to measure the rate of dissociation of [^{3}H]AcCh–receptor complexes formed at early times in the association reaction. When a *Torpedo* membrane suspension is incubated with 40 nM [^{3}H]AcCh for 10 s, only the high-affinity component of B_f' will be occupied (but not the low), and the rate of dissociation of [^{3}H]AcCh can then be measured as the rate of exchange of [^{3}H]AcCh for nonradioactive AcCh.

At 4 °C the dissociation of [^{3}H]AcCh–receptor complexes was readily measurable by the ultrafiltration assay since it was characterized by half-times of ~15 s. In Figure 6 is shown one experiment where the dissociation kinetics were determined for a *T. californica* membrane suspension 20 nM in α-toxin sites when the excess nonradioactive AcCh was added (1) 10 s after the addition of 40 nM [^{3}H]AcCh, (2) 30 min after the addition of 40 nM [^{3}H]AcCh, and (3) 30 min after the addition of 150 nM [^{3}H]AcCh. For each experimental condition the dissociation kinetics were characterized by a single rate constant (k_{dissoc} = 0.04 s^{-1}) equivalent to a half-time, $t_{1/2}$ = 17 s. Thus, the same rate constant for dissociation of [^{3}H]-AcCh–receptor complexes was observed under conditions when only the initial preexisting high-affinity receptor conformation had bound [^{3}H]AcCh and also when [^{3}H]AcCh was bound by the equilibrium receptor conformation.

The dissociation kinetics of [^{3}H]AcCh–receptor complexes at equilibrium were characterized more generally. The observed dissociation kinetics did not vary as the concentration of nonradioactive AcCh varied between 3 (20-fold excess over [^{3}H]AcCh) and 200 μM. In experiments with membrane suspensions prepared from four different *T. californica*, at 4 °C k_{dissoc} = 0.039 ± 0.006 s^{-1} ($t_{1/2}$ = 18 ± 3 s). Also, the rate of dissociation of [^{3}H]AcCh was the same when Carb or *d*-tubocurarine (1 mM) was the exchanging ligand as when AcCh was that ligand. These results indicate that high concentrations of agonists or antagonists do not modify the ligand binding properties of the cholinergic receptor equilibrated with [^{3}H]AcCh.

Reversibility of the Agonist-Induced Conformational Perturbation. In addition to characterizing the rate of dissociation of [^{3}H]AcCh–receptor complexes, we characterized the reversibility of the agonist-induced conformational perturbation of the membrane-bound *Torpedo* receptor following the removal of the stabilizing ligand. The basic protocol was as follows. A concentrated membrane suspension was equilibrated with an agonist at a concentration sufficient to occupy most receptor sites at equilibrium. That suspension was then diluted into a large volume of TPS so that the final

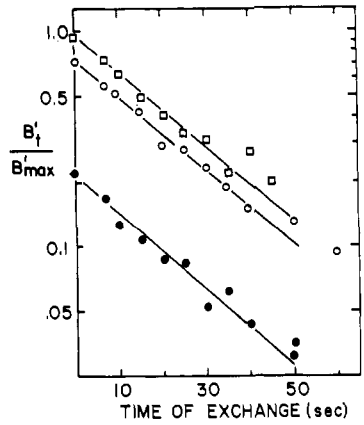


FIGURE 6: Kinetics of dissociation of [^{3}H]AcCh from *T. californica* membranes. The rate of dissociation of [^{3}H]AcCh was determined at 4 °C as the rate of exchange of [^{3}H]AcCh for nonradioactive AcCh. A membrane suspension 20 nM in α-toxin sites was allowed to react with 40 nM [^{3}H]AcCh for 10 s (●) or for 30 min (○) before nonradioactive AcCh (800 nM final concentration) was added to initiate the exchange. In a second experiment (□) a membrane suspension (20 nM in α-toxin sites) was equilibrated with 150 nM [^{3}H]AcCh before the addition of 3 μM nonradioactive AcCh. (Ordinate) [^{3}H]AcCh bound specifically to the receptor expressed as a fraction of the total number of sites. (Abscissa) Time after addition of nonradioactive AcCh. For each experiment the data includes results obtained from two separate mixing experiments. Dissociation of [^{3}H]AcCh receptor complexes prior to equilibrium was measured as follows. Equal volumes of a membrane suspension 40 nM in α-toxin sites and a solution containing 80 nM [^{3}H]AcCh were mixed in the rapid-mixing system and the total volume (46 mL) was collected. 10 s after mixing, an aliquot of that reaction mixture was filtered to determine the binding of [^{3}H]AcCh prior to the addition of nonradioactive AcCh. Between 13 and 15 s after mixing, 0.037 mL of a 1 mM AcCh solution was injected into the reaction mixture, and mixing was achieved by vigorous shaking. Aliquots (5 mL) were filtered at the indicated times after the addition of nonradioactive AcCh.

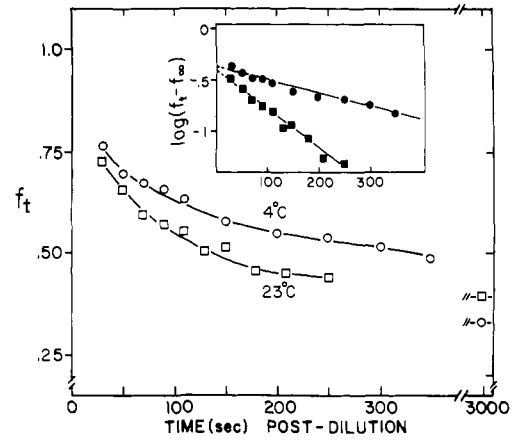


FIGURE 7: Reversibility of agonist-induced conformational perturbation of *T. californica* receptor. The reversibility of the agonist-induced conformational perturbation was examined at 23 °C (□, ■) and 4 °C (○, ●) by the following protocol. A *Torpedo* membrane suspension 2 μM in α-toxin sites was first equilibrated for 30 min with 3 μM Carb in TPS and then diluted 180-fold. At increasing time intervals after dilution, the amount of high-affinity receptor was determined by titration with [^{3}H]AcCh as follows. A 4.5-mL aliquot was mixed rapidly with 0.5 mL of a solution containing 300 nM [^{3}H]AcCh (final concentrations are 10 nM in α-toxin sites, 30 nM [^{3}H]AcCh, and 15 nM Carb), and the entire reaction mixture was filtered 5 s later. Control experiments established that 30 nM [^{3}H]AcCh and a 5-s reaction time was sufficient to occupy all binding sites existing in the high-affinity conformation and that the total number of binding sites remained constant during the recovery period. [^{3}H]AcCh bound specifically to the receptor was determined from the radioactivity retained on the filters. This value divided by the total number of AcCh binding sites is equal to the fraction (f_t) of receptor sites binding AcCh with high affinity at any given time (t) following dilution. (Inset) Semilogarithmic plot of the data.

agonist concentration was well below its equilibrium dissociation constant. Hence, when the system reequilibrates after dilution only a small fraction of the receptor sites would be occupied, and, if the agonist-induced conformational perturbation was reversible, the cholinergic receptor would relax back to its unliganded conformational state. The fraction (f_t) of receptor sites binding agonist with high affinity at any time after dilution was determined by an assay utilizing a short incubation with [^{3}H]AcCh. A knowledge of the dissociation constants of the rapid components of binding (Figure 5) permitted the selection of a concentration of [^{3}H]AcCh that was sufficient to saturate any unoccupied high-affinity binding sites (but not to occupy any low-affinity sites). In addition, the incubation time was chosen so that the binding to the high-affinity conformation would be complete but any perturbation of the conformational equilibrium by the titrating AcCh itself would be avoided. In practice it was found that for a suspension 10 nM in α-toxin sites, a 5-s reaction time with 30 nM [^{3}H]AcCh was sufficient to titrate the high-affinity sites.

In the experiments shown in Figure 7, Carb was the perturbing agonist. When a membrane suspension 2 μM in α-toxin sites and containing 3 μM Carb was diluted 200-fold, the fraction (f) of receptor sites binding AcCh with high affinity was equal to 0.8 immediately after dilution, and at a long time after dilution 30% of the sites bound agonist with high affinity. That final equilibrium value of f is larger than $f = 0.2$ characteristic of the *Torpedo* membranes upon first exposure to agonist because the residual Carb concentration following dilution still results in 10% receptor occupancy. The recovery was a first-order process characterized by half-times of 220 s at 4 °C and 80 s at 23 °C. Analysis of the concentration dependence of the conformational perturbation caused by Carb established that high concentrations caused a full conversion to high affinity ($f = 1.0 \pm 0.05$) and that the half-maximal effect was caused by a free concentration of Carb of 0.1 μM (data not shown) in good agreement with the equilibrium binding. Although the extent of conversion depended upon the Carb concentration employed, the recovery rate constant did not.

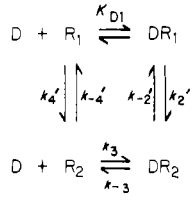
The recovery rate constant (k_{recov}) of the relaxation to the new equilibrium distribution of high- and low-affinity binding sites following removal of agonist was equal to 3×10^{-3} s^{-1} at 4 °C and 9×10^{-3} s^{-1} at 23 °C. At both temperatures k_{recov} was an order of magnitude smaller than k_{dissoc}, the rate of dissociation of [^{3}H]AcCh from the membrane-bound receptor, and, since Carb binds an order of magnitude more weakly than AcCh, it is reasonable to assume that the Carb dissociation rate constant is even higher than that of AcCh. Of particular mechanistic significance (see Discussion) is the observation that the value of k_{recov} [$(2.9 \pm 0.6) \times 10^{-3}$ s^{-1} in three experiments] was actually equal to the value of the rate constant characterizing the slowest phase of the association kinetics at low agonist concentration (Figure 4).

Reaction Mechanism. The simplest mechanism that adequately accounts for the data is presented in eq 1. This model assumes that there is a homogeneous population of receptors, each of which exists in two interconvertible conformations in the absence of any cholinergic ligand. The two preexisting conformations differ in the affinity with which they bind agonist. The observed biphasic concentration dependence of B_f' is a result of (relatively) rapid binding of agonist by the

Table I: Equilibrium and Rate Constants for Binding of [^{3}H] AcCh and [^{3}H] Carb by *Torpedo* Postsynaptic Membranes at 4 °C[a]

	[^{3}H] AcCh			
	T. marmorata[c]	*T. californica*	[^{3}H] Carb, *T. californica*	determination
K_{eq} (nM)	15 ± 2	8 ± 2	120 ± 20	
K_{D1} (nM)	1000	500	30000	concentration dependence of rapid reaction amplitude (B_f') and slow reaction rate constant (k_s)
K_{D3} (nM)	2.4	1.4	25	concentration dependence of rapid reaction amplitude (B_f')
k_3 ($M^{-1} \times s^{-1}$)		3×10^7		$k_3 = k_{-3}/K_{D3}$[b]
k_{-3} (s^{-1})		0.04		$k_{-3} = k_{dissoc}$ (Figure 6)
K_4'	5.6	4.5	4.5	concentration dependence of rapid reaction amplitude (B_f')
k_4' (s^{-1})	0.5×10^{-3}	0.5×10^{-3}	0.5×10^{-3}	$\lim_{D \to 0} k_s = k_4' + k_{-4}'$; $k_{-4}'/k_4' = K_4'$
k_{-4}' (s^{-1})	2.8×10^{-3}	2.3×10^{-3}	2.3×10^{-3}	
K_2'	0.013	0.014	0.0036	$K_2' = K_{D3}K_4'/K_{D1}$
k_2' (s^{-1})	0.18	0.06	0.15	concentration dependence of k_s
k_{-2}' (s^{-1})	0.0024	0.0008	0.54×10^{-3}	$k_{-2}' = K_2'k_2'$

[a] Parameters are determined according to the two-state model (eq 1) in which the receptor exists in two conformations, one binding agonist with low affinity (K_{D1}) and the other with high affinity (K_{D3}). [b] See also Boyd & Cohen (1980). [c] Data presented in Cohen & Boyd (1979).



$$K_{D1} = \frac{[D][R_1]}{[DR_1]}; K_{D3} = \frac{[D][R_2]}{[DR_2]}; K_2' = \frac{[DR_1]}{[DR_2]}; K_4' = \frac{[R_1]}{[R_2]} \quad (1)$$

two distinct receptor conformations, R_1 binding agonist with low affinity and R_2 binding with high-affinity. The slow relaxation rate constant, k_s, describes the conformational isomerization that follows the rapid phases of binding.

The applications of this model have been discussed in terms of pharmacological desensitization (Katz & Thesleff, 1957; Rang & Ritter, 1970) and enzyme mechanisms (Janin, 1973; Hammes & Wu, 1974). Lancet & Pecht (1976) provide an analytical expression for the dependence of k_s upon the rate and equilibrium constants of eq 1. The limiting value of k_s at low ligand concentration ($D \ll K_{D3}$) is equal to $k_4' + k_{-4}'$, the rate constant for isomerization of the unliganded receptor, while the limiting value of k_s at high concentrations ($D \gg K_{D1}$) is equal to $k_2' + k_{-2}'$. The rate constant (k_{recov}) for recovery to the low-affinity conformation upon removal of agonist is also equal to $k_4' + k_{-4}'$. Since k_s can approach $k_4' + k_{-2}'$ for intermediate ligand concentrations ($K_{D3} < D \ll K_{D1}$), k_s can be less than k_{recov} [as pointed out by Katz & Thesleff (1957) and Rang & Ritter (1970)]. We present here parameters of this model that fit the observed concentration dependence of B_f' and of k_s, and in the Discussion we present alternate, less satisfactory models.

Ligand association kinetics were calculated on a Data General Nova 3 computer by numerical integration of the differential equations specified by eq 1. A modified Euler's method was used. Numerical integration was necessary since for most experimental conditions no simplification of the reaction kinetics based upon an assumption of buffered concentrations was appropriate. The concentration dependence of k_s and B_f' were determined from the calculated values of B_t' from plots of log ($B_\infty' - B_t'$) against time.

The two-conformation reversible cycle (eq 1) is fully specified by three equilibrium constants and four rate constants, one for each reaction step. Knowledge of any three equilibrium constants determines the value of the fourth in such a thermodynamic cycle, and for each reaction step, the principle of detailed balance (Fowler, 1928) requires that $K = k_-/k_+$. The number of adjustable parameters was limited to four by the following considerations. (1) Of the four rate constants only two were varied, the isomerization rate constants k_2' and k_4'. The experimental data established that binding occurred rapidly, and it was thus plausible to assume that both ligand binding steps (K_{D1} and K_{D3}) were in rapid equilibrium relative to the receptor isomerization rates. Since k_{-3} was determined by the directly measured value of k_{dissoc}, a value of k_{+3} was calculated from K_{D3} and k_{-3}. (2) The three equilibrium constants were constrained to generate the independently determined equilibrium dissociation constant

$$K_{eq} = \frac{[D]([R_1] + [R_2])}{[DR_1] + [DR_2]} = \frac{K_{D3}(1 + K_4')}{1 + (K_{D3}/K_{D1})K_4'} \quad (2)$$

Thus only two equilibrium constants were varied independently.

The parameters listed in Table I, characterizing the interaction of [^{3}H]AcCh and [^{3}H]Carb with receptor of *T. californica*, were used to generate the solid curves in Figures 4 and 5. Also included are parameters characterizing the binding of [^{3}H]AcCh to membranes isolated from *T. marmorata* [data presented in Cohen & Boyd (1979)]. The model is able to reproduce reasonably well the observed data. The two receptor conformations differ in affinity for both agonists by 3 orders of magnitude, and comparison of the isomerization rate constants $k_{\pm 2}'$ and $k_{\pm 4}'$ reveals a selective effect of each agonist on the rate constant of the transition from the low- to the high-affinity conformation. For the unliganded receptor, k_4' is equal to 0.0005 s^{-1}, while k_2' is enhanced by at least 2 orders of magnitude. On the other hand, k_{-2}' and k_{-4}' differed by less than a factor of 5. The kinetics of binding of [^{3}H]AcCh to the membrane-bound receptor isolated from the two *Torpedo* species are reproduced by slightly different parameters. We wish to emphasize, however, the basic similarity of the results. There is no evidence that membranes prepared by similar procedures from the two fish differ significantly in their ligand binding properties.

Several comments are appropriate about the fit of the data by the cyclic model. (1) The cyclic reaction mechanism (eq 1) implies that K_4', k_4', and k_{-4}' are characteristic of the membrane-bound receptor itself and thus these values should be the same for all ligands. K_4' is determined by the concentration dependence of B_f', and $k_4' + k_{-4}'$ is determined by the limiting value of k_s at low ligand concentration. Both the [^{3}H]AcCh and the [^{3}H]Carb data are accounted for by the same values of K_4' and $k_{\pm 4}'$. (2) As predicted, the rate of dissociation of [^{3}H]AcCh–receptor complexes was characterized by the same rate constant, k_{dissoc} = 0.05 s^{-1} (4 °C), when ligand dissociation was measured at equilibrium or when

measured 10 s after mixing when only the sites binding AcCh rapidly and with high affinity were occupied (Figure 6). (3) The rate constant (k_{recov}) for the "recovery" to the low-affinity conformation upon removal of agonist was equal to $k_4' + k_{-4}'$ and an order of magnitude slower than k_{dissoc}.

The simple reaction mechanism (eq 1) reproduces the data reasonably well, but systematic deviations do exist in the fit for the interaction of [^{3}H]AcCh with *Torpedo* postsynaptic membranes. The concentration dependence of k_s is determined not only by the rate constants $k_{\pm 4}'$ and $k_{\pm 2}'$ but also by the dissociation constants K_{D1} and K_{D3}. Since for both AcCh and Carb, K_{D3} and K_4' were determined from the concentration dependence of B_f', K_{D1} was the only equilibrium constant adjusted in the fit of k_s. For AcCh, K_{D1} must be compatible with the concentration dependence of B_f' and k_s, but for Carb, it is determined only by the concentration dependence of k_s. From the data in Figure 5, K_{D1} for AcCh cannot be weaker than 1 μM, but in that case, values of k_2' and k_4' cannot be found that reproduce fully the available data concerning the concentration dependence of k_s (Figure 4). The calculated value of k_s agrees with the observed rates at the higher concentrations only if parameters are used that overestimate the rates at the lower concentration. Because of this deviation, we feel it is not appropriate to emphasize the uncertainty of the parameters based upon a least-squares goodness of fit. Further studies are in progress to obtain data at higher agonist concentrations to permit further refinement of the model.

Discussion

In this report we describe experimental procedures which permit an anlysis of the kinetics of binding of [^{3}H]AcCh and [^{3}H]Carb to nicotinic postsynaptic membranes isolated from *Torpedo* electric tissue. Kinetics of ligand binding on the second time scale were determined by the use of rapid-mixing procedures in conjunction with a novel ultrafiltration assay. The data provide clear evidence that upon initial exposure to agonist the membrane-bound nicotinic receptors from *Torpedo* must be characterized in terms of two receptor populations differing in their affinity for agonists.

We have shown that the data are well fit by a model assuming the existence of a single class of receptors that exist, in the absence of cholinergic ligands, in two interconvertible conformations, one binding agonist with low and the other with high affinity. We also considered alternate reaction mechanisms to determine whether other simple mechanisms could fit the data equally well. Models based on the assumption that only a single conformation binds AcCh initially cannot account for the observed high- and low-affinity components of B_f'. A possible explanation could be, however, that the *Torpedo* postsynaptic membranes contain a heterogeneous population of binding sites, either as a consequence of a true heterogeneity within the electroplaque such as junctional and extrajunctional receptors or as a result of the membrane isolation procedure. *Torpedo* nicotinic receptors extracted by detergent from membranes isolated by our normal procedure do appear heterogeneous in the sense that they exist in monomeric (9S) and dimeric (12S) forms (Chang & Bock, 1977; Hamilton et al., 1977; Neubig & Cohen, 1979).

Might the concentration dependence of B_f' reflect the existence of two receptors, R_α characterizing the 20% of the sites that bind agonist rapidly and with high affinity and R_β binding agonist weakly initially and then isomerizing to a high-affinity conformation (eq 3)? In this model the slow relaxation rate

$$\begin{aligned} D + R_\alpha &\rightleftharpoons DR_\alpha \\ D + R_\beta &\rightleftharpoons DR_\beta \rightleftharpoons DR_\beta' \end{aligned} \qquad (3)$$

constant k_s would reflect the isomerization $DR_\beta \rightleftharpoons DR_\beta'$. However, in such a model where the ligand dissociates only from a low-affinity conformation (DR_β), the rate of dissociation of drug–receptor complexes at equilibrium is limited by the slow conformational transition (Rang & Ritter, 1970; Janin, 1973). Thus k_{dissoc} cannot exceed k_{recov}, a prediction in disagreement with the observed data. Two further facts are difficult to reconcile with a simple model postulating binding site heterogeneity. (1) The rate of dissociation of [^{3}H]AcCh–receptor complexes when measured after exposure to low concentrations of [^{3}H]AcCh for 10 s would reflect the dissociation of DR_α, and it would be highly fortuitous for that rate to be the same as the one characterizing the dissociation of DR_β at equilibrium. (2) The limiting value of k_s at low agonist concentrations would be determined by the rate of isomerization of liganded receptors and would be expected to differ for AcCh and Carb.

The analysis presented here of the ligand binding properties of the membrane-bound nicotinic receptor of *Torpedo* can be compared with the results obtained by other techniques. Analysis of the effects of cholinergic ligands on the kinetics of binding of radiolabeled α-neurotoxins established the existence of a slow, reversible receptor conformational transition (Weber et al., 1975; Weiland et al., 1977; Quast et al., 1978; Barrantes, 1978; Weiland & Taylor, 1979). The concentration dependence of k_s and also a value for k_{recov} were estimated. No determination of the limit value for k_s at low ligand concentration was made, and thus it was not possible to determine whether that value was independent of ligand and equal to k_{recov}. Also, it was not possible to estimate k_{dissoc} for the rapidly reversible agonists. In those studies there is no agreement about the ability of the two-conformation reaction cycle to account for the data. Weiland et al. (1977) concluded that a reaction cycle involving two receptor conformations did account for the effects of cholinergic ligands on the kinetics of α-toxin binding. In particular they emphasized that for 0.3 μM Carb, k_s was less than k_{recov} in accord with the predictions of the cyclic model. The parameters they determined for the interaction of Carb with the *Torpedo* receptor agree reasonably well with those reported here, and some of the differences may be explained by the fact that their studies were carried out in the absence of divalent cations. In a similar study, however, Quast et al. (1978) concluded that parameters for Carb determined from the cyclic mechanism (eq 1) could not generate the correct equilibrium binding. We have observed no comparable discrepancy.

In a recent report Quast et al. (1979) utilized the fluorescence of the aromatic amine, ethidium bromide, in conjunction with stopped-flow techniques to analyze the ligand binding properties of the membrane-bound *Torpedo* receptor. They concluded that the *Torpedo* receptor exists in a single conformation in the absence of cholinergic ligands that contains two independent binding sites, one binding Carb with a dissociation constant of 1 μM and a second binding Carb with a dissociation constant of 1 μM and a second binding Carb with a dissociation constant $\sim$50 μM. The binding of each ligand is coupled to a conformational transition resulting in high-affinity equilibrium binding. Since this interpretation is different than ours, it is useful to compare the experimental observations. When the *Torpedo* membranes were pretreated with ethidium, subsequent addition of submicromolar concentrations of Carb resulted in a slow increased fluorescence, with half-times exceeding 60 s. The addition of higher Carb concentrations was characterized by an additional faster relaxation with half-times ranging between 4 s at 5 μM Carb and 0.4 s at 100 μM Carb as well as the slow relaxation. In contrast to our results, no rapid relaxation was detected in the

presence of low Carb concentration. That result is not as surprising as it appears for the following reason. If the detected fluorescence signal is proportional to receptor in a high-affinity conformation, the binding of Carb by a *preexisting* high-affinity conformation would result in no change of fluorescence, and, therefore, preexisting high-affinity receptor would not be detected. A second difference between the slow relaxation we report and that revealed by ethidium is seen in the concentration dependence of k_s. In our studies k_s decreased by 30% between 50 and 500 nM Carb and then increased by an order of magnitude as Carb increased to 5 μM. In the presence of ethidium the slow relaxation rate constant decreased by 50% as Carb varied between 0.5 and 5 μM. It is not possible to propose a simple model accounting for the agonist kinetics observed in the presence and absence of ethidium. Elliott & Raftery (1979) reported that high ethidium concentrations (apparent K_I = 25 μM) displaced the noncompetitive antagonist [^{3}H]histrionicotoxin bound to *Torpedo* membranes. We have found that in the absence of cholinergic ligands ethidium displaces [^{3}H]histrionicotoxin with an apparent K_I of 80 μM, but in the presence of 30 μM Carb the apparent K_I is 1 μM (Medynski & Cohen, 1980). Thus, ethidium may modify receptor conformational equilibria in a manner similar to other noncompetitive antagonists (Weiland et al., 1977; Heidmann & Changeux, 1979b; Blanchard et al., 1979; Boyd & Cohen, 1979; Cohen et al., 1980). It should be emphasized that the model accounting for the ethidium data is not compatible with our data concerning the values of k_s, k_{dissoc}, and k_{recov}.

While the description of the *Torpedo* conformational equilibria revealed by ethidium fluorescence differs from our conclusions, our results are in good agreement with the conclusions drawn by Heidmann & Changeux (1979a,b) from a study of the kinetics of binding of a fluorescent cholinergic agonist to receptor-rich membranes of *T. marmorata*. In that work three relaxation rates were detected and the data were well accounted for by the cyclic reaction mechanism. The only parameters of the model that are ligand independent are the values of K_4', k_4', and k_{-4}'. The reported value of K_4' agrees with ours. When compared at the same temperature, the rate constants were a factor of 5 higher than we determined. This difference reflects in part the fact that the reported concentration dependence of k_s at low ligand concentrations differs by a factor of 2–3 in the two studies. A primary advantage of the fluorescence technique is that it permits the determination of k_s at high agonist concentrations in order to demonstrate that k_s attains a limiting value independent of drug concentration. In the studies reported here we have not accumulated data at higher ligand concentration. Since the value of k_s at high ligand concentrations determines $k_{\pm 2}'$ while that at low concentrations determines $k_{\pm 4}'$, the two techniques are complementary.

The binding data here can be reasonably well accounted for by the cyclic reaction mechanism (eq 1). The concentration dependence of B_f' has been attributed to binding of agonist to two preexisting conformations. In the following report we utilize an automated ultrafiltration apparatus to measure ligand binding kinetics on the subsecond time scale to characterize the ligand binding attributed to the preexisting receptor conformations R_1 and R_2. After presenting an analysis of the rapid components of the ligand association kinetics, we will discuss the results in terms of processes of channel activation and receptor desensitization.

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Kinetics of Binding of [^{3}H]Acetylcholine to *Torpedo* Postsynaptic Membranes: Association and Dissociation Rate Constants by Rapid Mixing and Ultrafiltration†

Norman D. Boyd and Jonathan B. Cohen*

ABSTRACT: An automated rapid-mixing ultrafiltration apparatus was constructed to measure at subsecond times the kinetics of binding of radiolabeled cholinergic ligands to nicotinic postsynaptic membranes isolated from *Torpedo* electric tissue. The dissociation of [^{3}H]AcCh–receptor complexes at 7 and 23 °C was characterized by dissociation rate constants (k_{dissoc}) of 0.05 s^{-1} and 0.15 s^{-1}, respectively. The association kinetics at low concentrations of [^{3}H]AcCh were determined at 23 °C and found to be consistent with a model in which 20% of the AcCh binding sites preexist in a receptor conformation that binds AcCh with high affinity (K_D = 3 nM) and with a bimolecular association constant, $k_+ = 5.7 \times 10^7$ M^{-1} s^{-1}. Initial studies of the association kinetics at higher AcCh concentrations revealed a transient low-affinity binding step that occurred relatively slowly. In the presence of 0.8 μM [^{3}H]AcCh, this component of the association reaction was characterized by an experimental rate constant of 2 s^{-1}. The observed binding kinetics are discussed with reference to the processes of channel activation and receptor desensitization.

The free energy of ligand binding is utilized by nicotinic cholinergic receptors to control membrane permeability. In order to gain a better understanding of the mechanism involved, it is necessary to characterize receptor conformational equilibria in terms of ligand binding and to relate those conformations to the functional states of the ion channel. Nicotinic postsynaptic membranes isolated from *Torpedo* electric tissue provide a useful preparation for which it is possible to define both ligand binding and permeability response functions [reviewed in Heidmann & Changeux (1978)].

Measurement of the agonist-stimulated efflux of radioisotopes established that for the isolated *Torpedo* vesicles, as for intact cells, the nicotinic response entails an increased permeability to cations (channel opening: Popot et al., 1976; Miller et al., 1978; Neubig et al., 1979) that is transient in nature (receptor desensitization: Sugiyama et al., 1976; Bernhardt & Neumann, 1978). Recently, techniques have been developed (Neubig & Cohen, 1980a,b) to measure on the millisecond time scale the agonist-stimulated efflux of $^{22}Na^+$ and it is thus possible to define quantitatively agonist dose–response relations for both channel activation and receptor desensitization. Stopped-flow fluorescence techniques using the fluorescence of intrinsic membrane protein (Bonner et al., 1976; Barrantes, 1978), of cholinergic agonists (Heidmann & Changeux, 1979), and of noncompetitive antagonists (Grunhagen et al., 1977; Quast et al., 1979) have been introduced to characterize ligand binding kinetics and to identify receptor conformations that might be involved in channel activation.

In the preceding report (Boyd & Cohen, 1980) manual mixing and ultrafiltration techniques were used to quantify the kinetics of binding of [^{3}H]acetylcholine (AcCh)[1] and [^{3}H]carbamoylcholine (Carb) on the second time scale. We report here a further analysis of the kinetics of agonist binding to membrane-bound *Torpedo* receptor. An automated rapid-mixing ultrafiltration apparatus is described that permits the measurement of [^{3}H]AcCh binding kinetics with a temporal resolution of 0.1 s. The observed dependence of the association kinetics upon the concentration of AcCh and receptor provides direct evidence that 20% of the AcCh binding sites preexist in a high-affinity receptor conformation. The limitations of a reaction mechanism involving only two conformational states are discussed in terms of the available binding and flux data.

Materials and Methods

Biological Materials and Chemicals. Nicotinic postsynaptic membranes were isolated from *Torpedo californica* electric tissue by the procedure of Sobel et al. (1977). All chemicals and radiochemicals were from sources described in the previous

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[1] Abbreviations used: AcCh, acetylcholine; TPS, *Torpedo* physiological saline (250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM sodium phosphate, pH 7.0, and 0.02% NaN_3).

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