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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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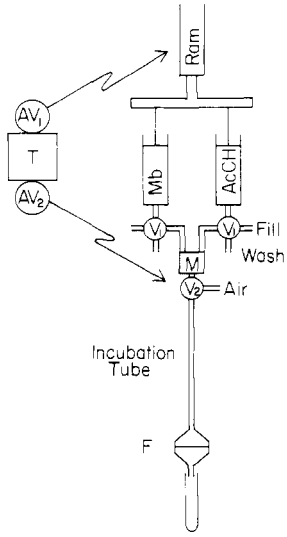

FIGURE 1: Block diagram of rapid-mixing ultrafiltration apparatus. For initiation of the reaction, an electrically operated solenoid air valve (AV_1) is opened and the pneumatic ram forces the contents of the two reactant syringes through an eight-jet tangential mixer (M, Durrum Instruments, Palo Alto, CA) and then via a pneumatic three-way slide valve (V_2, Durrum) into a Teflon incubation tube (usually 1-mL capacity, inner diameter = 1.5 mm, length = 56 cm). The incubation tube is attached directly to a polypropylene disk filter holder (F, Swinnex 25 mm, Millipore Corp., Bedford, MA). The mixture resides in the incubation tube until the air valve (AV_2) which controls V_2 is electronically activated by timer T altering the position of slide valve V_2 so that the incubation tube is closed to M and opened to 27 psi air pressure. This positive pressure forces the reaction mixture out of the incubation tube and through the glass filter (Whatman GFF) contained within filter holder F. Four-way manual valves (V_1) permit access to the reactant syringes and mixer system for sample loading and washing.

report (Boyd & Cohen, 1980). [^{3}H]AcCh binding studies were carried out in *Torpedo* physiological saline (TPS, containing 250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM sodium phosphate, pH 7.0) containing 0.1 mM diisopropyl phosphorofluoridate. All studies were at 23 °C unless otherwise noted.

Automated Rapid-Mixing and Ultrafiltration Apparatus. An automated rapid-mixing ultrafiltration apparatus was constructed to study on the subsecond time scale the kinetics of binding of radiolabeled ligands to *Torpedo* postsynaptic membranes. A block diagram of the apparatus is presented in Figure 1. A pneumatic ram is used to mix rapidly the contents of two syringes, one containing a *Torpedo* membrane suspension and the other [^{3}H]AcCh. The reaction mixture is delivered to a Teflon incubation tube, and the binding reaction is terminated at a predetermined time by the activation of an automatic three-way slide valve (V_2) which causes the contents of the incubation tube to be forced rapidly by positive pressure through an ultrafiltration unit. The membrane suspension and bound [^{3}H]AcCh are retained on the filter, while free [^{3}H]AcCh is collected in the filtrate.

The rapid-mixing component of the apparatus has been adapted from conventional stopped-flow technology (Gibson & Milnes, 1964) and consists of two parallel thermostated syringes connected to an eight-jet tangential mixer. The ram operating pressure was usually 50 psi, and, in separate experiments utilizing this mixing system in conjunction with a chemical quench mixer, adequate mixing on the millisecond time scale has been established (Neubig & Cohen, 1980b).

Glass fiber filters (Whatman GFF) have a high fluid flow rate and good retention capacities and thus were used for the positive pressure filtration necessary in the automated rapid-mixing ultrafiltration rig. *Torpedo* membrane suspensions (40 nM in α-toxin sites) equilibrated with saturating concentrations

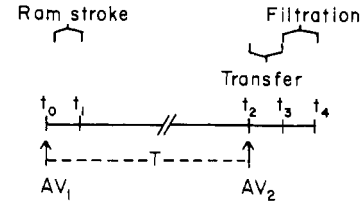

FIGURE 2: Temporal sequence for rapid-mixing ultrafiltration. Activation of air valve AV_1 (at time t_0) directs air pressure (50 psi) to the ram. Ram motion is initiated after a 30-ms lag, and for an 0.82-mL reaction mixture the ram stroke is completed at $t_1 = 108 \pm 9$ ms. The reaction mixture resides in the incubation tube for the desired time, and then air valve AV_2 is activated at t_2. Following activation of AV_2, there is a lag before the filter is wetted (t_3). The lag ($t_3 - t_2$) is reproducible and is equal to 190 ± 5 ms. Filtration begins at t_3 and is completed at t_4. For an 0.82-mL reaction mixture $t_4 - t_3$ is 160 ms while for a 2.14-mL volume it is 420 ms. Filtration times were the same as 4 and 23 °C. The time interval between activation of AV_1 and AV_2 ($T = t_2 - t_0$) is defined by the electronic timer, but the actual reaction is longer because of the time lags and finite filtration times. Estimated reaction time = $T + (t_3 - t_2) + (t_4 - t_3) - t_1$. For an 0.82-mL reaction mixture that value exceeds T by 240 ms, while for a 2.14-mL reaction volume, the reaction time is approximately T + 420 ms.

of [^{3}H]AcCh (300 nM) were filtered at pressures between 15 and 35 psi to test the retention properties of the GFF filters under positive pressure. The [^{3}H]AcCh bound specifically to the receptor was determined from the total [^{3}H]AcCh retained on the filters by the use of control membrane suspensions with receptor sites occupied by α-bungarotoxin as previously described (Boyd & Cohen, 1980). Although the nonspecific retention of [^{3}H]AcCh showed a small dependence upon filtration pressure (~10% reduction over the range examined), the [^{3}H]AcCh bound specifically to the membrane-bound receptor was independent of filtration pressure and equal to the value determined by vacuum filtration (or ultracentrifugation). It is desirable to use the maximum possible filtration pressure in order to minimize the filtration time, but we have observed that pressures >27 psi are not feasible. Higher pressures result in the formation of an aerosol when the reaction mixture is forced out of the incubation tube, and the droplets formed result in variable filtration volumes. Therefore, a filtration pressure of 27 psi was used routinely.

The resolution attainable in the analysis of ligand binding kinetics by the use of rapid-mixing and ultrafiltration is determined by the precision with which short reaction times can be measured. The technique requires the transfer of reaction mixture to an incubation tube and then from that tube into the filter holder and through the filter. The various temporal segments are indicated in Figure 2. The time defined by the electronic timer is the interval T between the activation of air valve AV_1 initiating the ram stroke and the activation of air valve AV_2 initiating filtration. The motion of the ram was monitored by the change in resistance with time of a linear potentiometer connected in parallel with the ram. To follow the filtration process, we modified a filter holder to include two wire leads on the support under the filter. By inclusion of these leads in an appropriate electronic circuit, it was possible to determine when the filter was first wetted and also when the last of the solution was filtered. After activation of AV_1 there was a lag of 30 ms before the initiation of the ram stroke, and after activation of AV_2 there was a lag of 190 ± 5 ms before the filter was wetted. While those lags were independent of reaction volume, the duration of the ram stroke and the filtration process itself were volume dependent. For an 0.82-mL reaction mixture, those times were 80 and 160 ms, respectively, while for a 2.14-mL volume, the times were 160 and 420 ms, respectively. When these factors are taken into account, the mean reaction time for an 0.82-mL volume

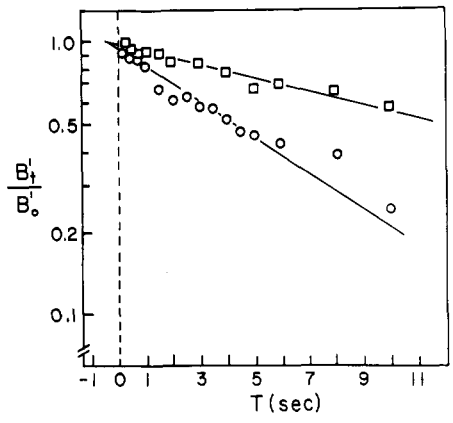

FIGURE 3: Kinetics of dissociation of [^{3}H]AcCh receptor complexes at 7 ± 1 °C (□) and at 23 °C (○). The rate of dissociation was determined as the rate of exchange of [^{3}H]AcCh for nonradioactive AcCh. A membrane suspension (100 nM in α-toxin sites) equilibrated with 300 nM [^{3}H]AcCh in TPS was mixed rapidly with an equal volume of a TPS solution containing 40 μM nonradioactive AcCh, and the exchange reaction was terminated by ultrafiltration at the indicated time after mixing. B_t' is the amount of [^{3}H]AcCh bound specifically to the receptor at time T; B_0' is the amount of [^{3}H]AcCh bound specifically to the receptor at zero time, determined by mixing the membrane suspension with a TPS solution containing no AcCh. (Abscissa) the time interval T between the initiation of the mixing process (activation of AV_1) and the initiation of filtration (activation of AV_2).

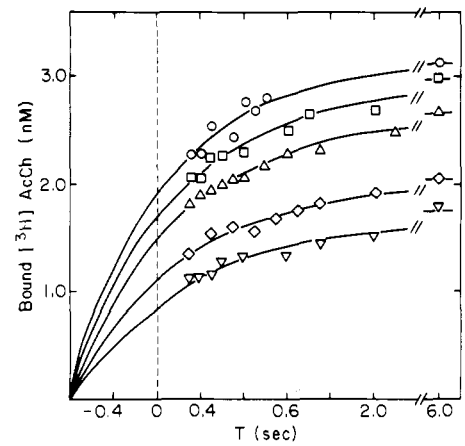

FIGURE 4: Kinetics of binding of low concentrations of [^{3}H]AcCh to *Torpedo* membranes at 23 °C: ligand excess. For reaction mixtures containing [^{3}H]AcCh concentrations in excess of the concentration of high-affinity binding sites, volumes of 2.14 mL were filtered in the automated rapid-mixing ultrafiltration apparatus, and [^{3}H]AcCh bound specifically to the nicotinic receptor (B_t') was determined from radioactivity retained in the filter as described under Materials and Methods. The reaction mixture contained 20 nM α-toxin sites (3.8 nM AcCh sites in a high-affinity conformation) and 3.6 (▽), 5.6 (◇), 6.8 (△), 8 (□), and 9.5 (○) nM [^{3}H]AcCh. (Abscissa) Time T at which air valve AV_2 was activated to initiate filtration. (Solid curves) B_t' calculated for K_D = 2.3 nM, $k_- = 0.23\ s^{-1}$, $k_+ = k_-/K_D = 10 \times 10^7\ M^{-1}\ s^{-1}$, 18.7% of the α-toxin sites in the high-affinity conformation, and a reaction time equal to T + 0.8 s (see text).

is estimated to exceed the timer interval T by ~240 ms, and for a 2.14-mL volume, the reaction time exceeds T by ~420 ms (see also Figure 2). These calculations cannot be exact, and for this reason we present the ligand binding kinetics under Results in terms of T and discuss explicitly the effective lags determined from the ligand binding data.

Results

Kinetics of Dissociation of [^{3}H]AcCh from Membrane-Bound Torpedo Receptor. The kinetics of dissociation of [^{3}H]AcCh–receptor complexes at equilibrium is well characterized by a single unimolecular rate constant, k_{dissoc} = 0.04 s^{-1} at 4 °C (Boyd & Cohen, 1980). We used this dissociation reaction to assess the performance of the automated rapid-mixing ultrafiltration apparatus. In particular, we wished to obtain an experimental definition of the effective reaction times when the reaction is terminated by ultrafiltration a fraction of a second after mixing. In Figure 3 the amount of [^{3}H]AcCh bound specifically to the receptor at increasing time intervals after addition of excess nonradioactive AcCh is shown for exchange reactions at 7 and 23 °C. At 7 °C the half-life ($t_{1/2}$ = 12 s) was a factor of 2.5 greater than that at 23 °C ($t_{1/2}$ = 4.5 s). Furthermore, at both temperatures the observed dissociation kinetics extrapolate to the independently determined initial specific binding (B_0') at a time of −0.3 ± 0.1 s, indicating that the actual incubation time is slightly greater than the electronically determined time interval (T) and is equal to T + 0.3 s. The additional reaction time observed experimentally is thus in agreement with the value calculated from the control experiments presented under Materials and Methods. The time increment is dependent upon the reaction volumes filtered, and, when larger volumes (2.14 mL) were utilized, the dissociation reaction at 23 °C was characterized by the same rate constant but the time increment was equal to 0.6 ± 0.2 s.

In experiments with membranes prepared from four fish and used within 15 days of the initial isolation, >90% of the [^{3}H]AcCh–receptor complexes dissociated according to a single, temperature-dependent, first-order rate process characterized by a rate constant, k_{dissoc} = 0.15 ± 0.03 s^{-1} (23 °C). We have also observed that for membrane suspensions stored for longer than 1 month after preparation, as much as 40% of the drug–receptor complexes dissociate with a rate constant ($k_{dissoc} \simeq 2\ s^{-1}$ at 23 °C) considerably higher than that normally observed, while the rate constant for dissociation of the remaining 60% was still 0.15 s^{-1}.

Association Kinetics at Low [^{3}H]AcCh Concentrations. In contrast to the relatively straightforward kinetic behavior of the dissociation reaction, the association reaction is more complex and is characterized by at least three distinct components. The kinetics of the slowest phase which involved slow conformational transitions of the receptor were examined in the previous report (Boyd & Cohen, 1980). Although a kinetic analysis of the more rapid phases was not possible, the total binding that occurred rapidly could be estimated. The concentration dependence of this binding reflected the presence of both high- and low-affinity components. The automated rapid-mixing ultrafiltration apparatus was therefore used to examine the kinetics of these rapid processes. We first examined the rapid binding reaction at low [^{3}H]AcCh concentrations so that only interactions with the high-affinity binding site would be significant.

Binding kinetics were determined for [^{3}H]AcCh concentrations varying between 4 and 12 nM and for a total concentration of receptor binding sites of 20 nM (~4 nM in high-affinity sites). Under these conditions it was necessary to filter large reaction volumes (2.14 mL) to obtain adequate definition of the [^{3}H]AcCh association kinetics. In Figure 4, specifically bound [^{3}H]AcCh is shown as a function of time for several AcCh concentrations. As before, the precisely defined time interval t is that between the activation of the two air valves, and not the full reaction time. The observed binding reactions are all characterized by half-times of 1 s or less. The binding at 6 s is expected to be a measure of the total amplitude of the high-affinity binding only, since at these low concentrations of AcCh and this short reaction time additional contributions due to binding from low-affinity components and the slow conformational isomerization step are not significant. This was confirmed by analyzing the concentration dependence of this amplitude by a double-reciprocal

plot which indicated that the fraction of α-toxin sites occupied was equal to 0.19 ± 0.01 and that the binding was characterized by a dissocation constant (K_D) equal to 2.5 ± 0.2 nM.

The solid curves in Figure 4 are the association kinetics calculated for a simple bimolecular interaction of [^{3}H]AcCh with that fraction of high-affinity sites by using a bimolecular rate constant, $k_+ = 1 \times 10^8$ M^{-1} s^{-1}, and a dissociation rate constant, $k_- = 0.23$ s^{-1}. These curves reproduced the experimental data reasonably well provided the actual reaction time was equal to the air valve interval (T) plus 0.8 s. Thus, the rate constants that reproduce the observed association kinetics are in reasonable agreement with the rate constant k_{dissoc} = 0.16 s^{-1} determined independently for that membrane suspension from the dissociation of [^{3}H]AcCh receptor complexes at equilibrium (Figure 3) and with the bimolecular rate constant calculated from the overall reaction equilibrium constant. The 0.8-s lag time was also consistent with the value 0.6 ± 0.2 s determined for the same reaction volume from the rate of dissociation of [^{3}H]AcCh–receptor complexes.

The preceding results provide strong evidence in support of the interpretation that the interaction of AcCh with its high-affinity binding site is a bimolecular association process. However, during the process of ultrafiltration, the membrane suspension is concentrated within the filter, and, if the receptors are still capable of binding AcCh, the entrapped receptors might do so at an accelerating rate until the filtration was terminated. In order to determine the importance of this effect in generating a possible artifact in the determination of k_+, we studied the reaction under conditions where the receptor concentration was higher than that of AcCh and thus the rate would be more sensitive to increases of receptor concentration.

The kinetics of binding of reaction mixtures containing low concentrations of [^{3}H]AcCh and excess receptor can be followed simply by determining the concentration of free [^{3}H]-AcCh collected in the filtrate as a function of time. Since only small volumes of filtrate are necessary to accurately determine the concentrations of free [^{3}H]AcCh, reaction volumes of 0.8 mL are adequate, and the large reaction volumes and resulting extended filtration times necessary in the experiments under conditions of [^{3}H]AcCh excess can be avoided. In Figure 5 is shown an experiment in which the reaction mixtures contained 2.9 nM [^{3}H]AcCh and receptor concentrations varying between 40 and 300 nM. A rapid decrease of [^{3}H]AcCh was observed, with both the rate and amplitude dependent upon receptor concentration. Also included are data obtained for a control experiment in which the free [^{3}H]AcCh was measured at different times for membranes treated with α-bungarotoxin. Since no variation of free AcCh was seen in that control, we conclude that the nonspecific interaction of [^{3}H]AcCh with either the *Torpedo* membranes or the glass fiber filters does not contribute significantly to the observed kinetics.

The observed association kinetics were analyzed in a manner analogous to the preceding series of experiments where the concentration of [^{3}H]AcCh exceeded that of high-affinity receptor sites. In order to do this, we had to determine the fraction of α-toxin sites binding AcCh with high affinity in a separate experiment, since under conditions of receptor excess only a small percentage of high-affinity sites is occupied at the end of this binding reaction. Those parameters were therefore determined for this membrane suspension by incubating the membranes with various concentrations of [^{3}H]-AcCh for 5 s. A double-reciprocal analysis of that data indicated that at 23 °C, 15 ± 1% of the receptor sites preexisted in the high-affinity conformation (K_D = 3.0 ± 0.4 nM). The

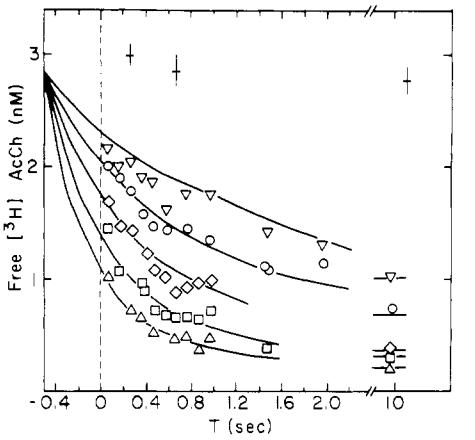

FIGURE 5: Kinetics of binding of low concentrations [^{3}H]AcCh to *Torpedo* membranes at 23 °C: receptor excess. For reaction mixtures containing concentrations of binding sites in excess of AcCh, the binding kinetics were monitored from the change of free AcCh (F_t). 0.82-mL samples were filtered in the rapid-mixing ultrafiltration apparatus, and F_t was determined from aliquots of the filtrate. The reaction mixture contained 2.9 nM [^{3}H]AcCh and 42 (▽), 74 (○), 105 (◇), 158 (□), and 210 (△) nM α-toxin sites. In control experiments (+), membranes pretreated with α-BgTx were used, and no significant variation of F_t was detected. (Abscissa) Time T at which air valve AV_2 was activated to initiate filtration. Solid curves were calculated for K_D = 3 nM, k_- = 0.16 s^{-1}, $k_+ = k_-/K_D = 5.3 \times 10^7$ s^{-1}, 15.4% of the α-toxin sites in the high-affinity conformation, and a reaction time equal to T + 0.5 s (see text).

kinetics of binding of [^{3}H]AcCh to this site by a simple bimolecular pathway were calculated by using a bimolecular rate constant, $k_+ = 5.3 \times 10^{-1}$ M^{-1} s^{-1}, and a dissociation constant, $k_- = 0.16$ s^{-1}. The calculated kinetic profiles, which are shown by the solid curves in Figure 5, reproduce the experimental data quite well provided the extra incubation time, Δt, is equal to 0.5 s. Since the minimal value of Δt for an 0.82-mL reaction volume determined from the dissociation experiments, reactions in which there is no dependency of the experimental rate constant on receptor concentration, is 0.3 ± 0.1 s, it appears that even under conditions of receptor excess, where the experimental rate constant is highly dependent upon receptor concentration, the receptor concentration effect inherent in ultrafiltration produces only small effects.

Determination of k_+ and k_- from High-Affinity Association Rates. To determine, from the observed kinetics, values of both k_+ and k_- that are independent of a knowledge of Δt, we analyzed the high-affinity binding kinetics observed in experiments with membrane suspensions from three fish, for both conditions of AcCh excess (as in Figure 4) and receptor excess (Figure 5), in terms of a semilogarithmic plot of reaction extent against time. The pseudo-first-order rate constants (k_{expt}) for each experiment were then determined by a linear least-squares analysis of these plots. For a bimolecular association process under pseudo-first-order conditions, the experimental rate constant, $k_{expt} = k_+C + k_-$, where C is the concentration of the reactant in excess, k_+ the bimolecular rate constant, and k_- the dissociation rate constant. The observed rate constants were therefore plotted in Figure 6 as a function of the concentration of AcCh (for excess AcCh) or high-affinity receptor (for excess receptor). When analyzed by linear least squares, the slope (k_+) was equal to (5.7 ± 0.7) × 10^7 M^{-1} s^{-1} and the intercept (k_-) was equal to 0.23 ± 0.07 s^{-1}. The dissociation constant calculated from these rate constants agrees with that determined from the reaction amplitudes.

Association Kinetics at High [^{3}H]AcCh Concentration. The rapid interaction of the membrane-bound receptor with [^{3}H]AcCh involves not only the high-affinity binding reaction

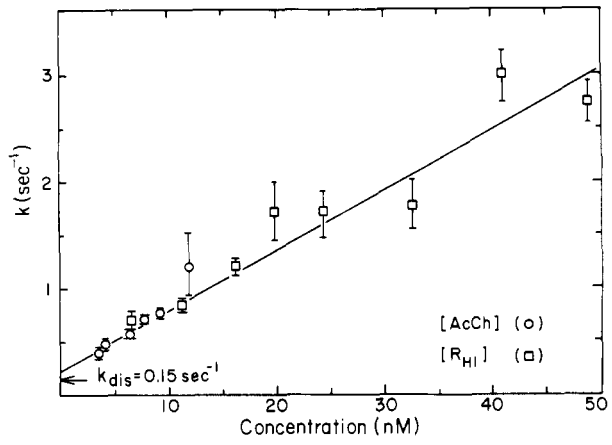

FIGURE 6: Dependence of experimental rate constant for high-affinity binding step upon concentration of AcCh and of receptor. The rapid component of [^{3}H]AcCh association kinetics was determined at 23 °C by rapid mixing and ultrafiltration under conditions of AcCh excess (as in Figure 4) or receptor excess (as in Figure 5). For each reaction, an experimental rate constant (k_{expt}) was determined from the slope of a plot of log ($B_\infty' - B_t'$) as a function of time by unweighted least-squares regression. (Abscissa) Concentration of reactant in excess [AcCh (○) or high-affinity receptor sites (□)]. (Solid line) Weighted linear least-squares regression line characterized by a slope (k_+) equal to $(5.7 \pm 0.7) \times 10^7$ M^{-1} s^{-1} and a y intercept (k_-) equal to 0.23 ± 0.07 s^{-1}. Also indicated is the rate of dissociation of [^{3}H]AcCh–receptor complexes at equilibrium (k_{dissoc} = 0.15 s^{-1}).

but also a transient low-affinity component. The automated ultrafiltration apparatus was employed to characterize at second and subsecond times this low-affinity binding reaction. In Figure 7 the results of an experiment are shown in which the [^{3}H]AcCh association kinetics have been determined at 23 °C for a reaction mixture containing 500 nM binding sites and 800 nM [^{3}H]AcCh. The automated apparatus was used to measure the binding for reaction times varying between 0.2 and 9 s, and manual techniques were used for times between 6 s and equilibrium. The equivalence of the two techniques is demonstrated here and in other similar experiments for the intermediate times. The slow binding which comprises 32% of the reaction amplitude is characterized by a half-time of 12 s.

The binding occurring during the rapid processes was analyzed by first subtracting the contribution of this slow phase from the observed specific binding (B_t'). In the inset of Figure 7 is a plot of log $[(B_f' - B_t')/B_f']$ as a function of time where B_f' is the total binding that occurs rapidly. Since binding involving the interaction of AcCh and the high-affinity site has been defined by a bimolecular rate constant of 5×10^7 M^{-1} s^{-1}, this step would be characterized by a half-time of ~20 ms under these experimental conditions and would thus be completed by the first experimental data point. The observed intermediate binding kinetics are due therefore to the low-affinity association reaction and are characterized by an experimental rate constant, $k_{int} \simeq 2.0$ s^{-1}. For the reaction volume (0.82 mL) used, the zero time is estimated to be about −0.4 s, and inspection of the observed binding kinetics (Figure 7, inset) indicates that the data are compatible with ~20% of the binding sites being occupied extremely rapidly.

The analysis of the ligand association kinetics shown in Figure 7 is clearly approaching the limit of resolution of the automated ultrafiltration apparatus. In a similar experiment at a lower concentration of [^{3}H]AcCh (150 nM) and of binding sites (50 nM), the intermediate phase of the binding was characterized by a smaller rate constant, $k_{int} \simeq 0.35$ s^{-1} (data not shown). Under these conditions, the lack of precision in the definition of the actual reaction time was less significant,

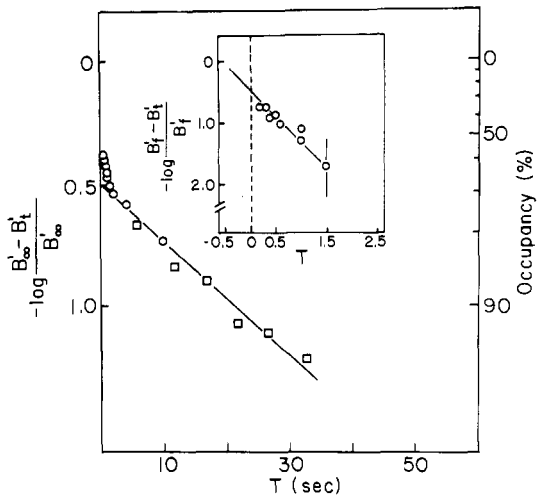

FIGURE 7: Kinetics of binding of [^{3}H]AcCh to *Torpedo* membranes at 23 °C: low-affinity component. The final reaction mixture contained 500 nM α-toxin sites and 800 nM [^{3}H]AcCh in TPS, and association kinetics were determined with an automated rapid-mixing ultrafiltration apparatus (○) or with a manual apparatus (□). [^{3}H]AcCh bound specifically to the receptor (B_t') was determined from [^{3}H]AcCh retained on the filter. For these reaction conditions, [^{3}H]AcCh bound at equilibrium (B_∞') was equal to the site concentration. The slowest component of the association reaction was characterized by a half-time of 12 s, and its amplitude (B_s') was equal to $0.3B_\infty'$. The amplitude of binding occurring rapidly (B_f') which is equal to $B_\infty' - B_s'$ was equal to $0.7B_\infty'$. (Abscissa) T, the time interval between the initation of the mixing (activation of air valve AV_1) and initiation of filtration (activation of AV_2). The actual reaction time is equal to $T + 0.4$ (see text). (Inset) Semilogarithmic plot of the rapid component of the observed association kinetics as a function of T. The solid line indicates a $t_{1/2}$ for the reaction of 0.35 s (k = 2.0 s^{-1}).

but no increase in the accuracy of the rate constant determination was possible since the lower AcCh concentration resulted in a smaller reaction amplitude for the intermediate phase. Thus, these experiments demonstrate the existence of a transient low-affinity binding step and provide data from which rate constants can be estimated. However, any detailed analysis depends upon the use of an apparatus with greater temporal resolution.

Discussion

In this report we describe the use of an automated rapid-mixing ultrafiltration apparatus to measure on the subsecond time scale the kinetics of binding of [^{3}H]AcCh to *Torpedo* postsynaptic membranes. Because the use of the apparatus involves the mechanical transfer of reactants to an incubation tube and then passage through the filter chamber, we described in detail the duration of those processes and the determination of the actual reaction time from an analysis of the kinetics of dissociation of [^{3}H]AcCh receptor complexes at equilibrium. Control experiments established that the duration of the fluid transfer operations was independent of temperature between 4 and 23 °C. The dissociation reaction was therefore characterized at 4 and 23 °C since both reactions must extrapolate to the same initial binding at zero time. For reaction volumes of 0.82 mL, the actual reaction time exceeds by 0.3 ± 0.1 s the time at which filtration is initiated, while for a volume of 2.14 mL, that time interval is 0.6 ± 0.2 s.

In the previous report (Boyd & Cohen, 1980) reaction kinetics were analyzed for which the rate constants were independent of receptor concentration: conformational isomerization rates and the rate of dissociation of drug–receptor complexes. The rate of binding of ligands will usually follow a second-order reaction law and depend upon both AcCh and

receptor concentration. Ultrafiltration inevitably results in a complex increase in the binding site concentration during the filtration process, and such a perturbation of receptor concentration could potentially complicate the analysis of bimolecular association kinetics.

The kinetics of binding of [^{3}H]AcCh to the fraction of receptor sites which bind AcCh rapidly and with high affinity were studied in detail to determine (1) whether ultrafiltration can be used to characterize rapid bimolecular association kinetics and (2) whether the parameters determined could provide direct evidence for the mechanistic model suggested by our previous studies on a slower time scale. With the techniques available, it was possible to determine ligand binding at times >0.3 s after mixing. Shorter time intervals were not possible probably because of a finite ultrafiltration time. However, the analysis of [^{3}H]AcCh association kinetics under conditions of excess AcCh (Figure 4) or receptor excess (Figure 5) confirm the previous results and establish that ultrafiltration can be used to characterize bimolecular association rate constants. At 23 °C the high-affinity conformation of the nicotinic receptor in the *Torpedo* membranes binds AcCh with $k_+ = 5.7 \times 10^7$ M^{-1} s^{-1} and $k_- = 0.23 \pm 0.07$ s^{-1}.

The observed bimolecular rate constant (k_+) for the binding of AcCh by the high-affinity conformation is within the range of values expected for diffusion-controlled reactions (Gutfreund, 1972). Also, the dissociation constant reflects an exceptionally high-affinity interaction for a ligand as small as AcCh. In fact, there are as yet no known small agonists or antagonists that are bound with higher affinity by that conformation. Thus the high-affinity conformation must be considered an optimal AcCh binding conformation. Comparable studies for ligand association kinetics are available for only one other ligand, the fluorescent agonist [β-[(5-dimethylamino-1-naphthalenesulfonamido)hexanoyloxy]-ethyl]trimethylammonium bromide (DNS-C_6-Chol) (Heidmann & Changeux, 1979). It is striking that both the association and dissociation rate constants for that ligand are within a factor of 2 of those reported here for AcCh. It is interesting to speculate that it is the acetylcholine moiety of the fluorescent agonist that contributes to the binding specificity and that the aromatic fluorophore may contribute very little to the interaction with the receptor in the high-affinity conformation.

The high-affinity conformation characterized here is the receptor conformation stabilized by the agonist at equilibrium, and thus it is presumably involved not in channel activation but in desensitization. The rate constant of the isomerization of liganded receptors from a low-affinity to high-affinity conformation is the same as the rate constant for desensitization of the flux response in cells in culture (Sine & Taylor, 1979), and we have found the same to be true in the *Torpedo* vesicles (N. D. Boyd, R. R. Neubig, and J. B. Cohen, unpublished observations). The study of ligand binding associated with channel activation, however, requires the analysis of the transient low-affinity binding of agonists.

The transient low-affinity binding observed in this report is probably related to signals detected by spectroscopic techniques. For a reaction mixture containing 500 nM binding sites and 800 nM AcCh, the intermediate kinetics were characterized by a half-time of 0.3 s (Figure 7). In the presence of micromolar concentrations of AcCh, the rapid quenching of protein fluorescence was characterized by a half-time ~0.1 s (Bonner et al., 1976), and the rapid increase of quinacrine or ethidium fluorescence (Grunhagen et al., 1977; Quast et al., 1979) was characterized by similar half-times, although the data have been interpreted quite differently.

Although sufficient data have not been accumulated to define precisely the kinetic behavior of the transient low-affinity binding, several factors lead us to conclude that the intermediate binding is not directly related to the opening of ion channels in the *Torpedo* vesicles. At 800 nM [^{3}H]AcCh, that binding is characterized by a rate constant, $k \simeq 2$ s^{-1}, but, when the kinetics of $^{22}Na^+$ efflux from the *Torpedo* vesciles are measured, there is no evidence for such a slow rate of opening of channels [Neubig & Cohen (1980a,b) and unpublished observations]. Also, the apparent affinities for channel activation determined in those flux studies for AcCh and Carb are 30 μM and 600 μM, respectively, a factor of 20–30 greater than the transient low-affinity binding detected by several different kinetic techniques (Weiland et al., 1977; Barrantes, 1978; Boyd & Cohen, 1980). Thus it appears that the low-affinity binding associated with the intermediate kinetics cannot be related simply to the process of channel activation, and further studies are necessary to provide a quantitative description of the conformational state of the receptor involved in ion transport.

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