Sudmeier, J. L., Evelhoch, J. L., & Jonsson, N. B.-H. (1980) *J. Magn. Reson. 40*, 377–390.
Vaughn, J. D., Mughrabi, Z., & Wu, E. C. (1970) *J. Org. Chem. 35*, 1141–1145.
Watanabe, K., Matsuda, T., & Sato, Y. (1981) *Biochim. Biophys. Acta 667*, 242–250.
Weber, E., Papamokos, E., Bode, W., Huber, R., Kato, I., & Laskowski, M., Jr. (1981) *J. Mol. Biol. 149*, 109–123.

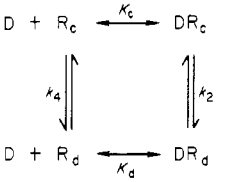
Conformations of *Torpedo* Acetylcholine Receptor Associated with Ion Transport and Desensitization†

Richard R. Neubig, Norman D. Boyd, and Jonathan B. Cohen*

ABSTRACT: Rapid-mixing techniques have been used to measure under identical conditions both the kinetics of binding of [^{3}H]acetylcholine (AcCh) to nicotinic receptors in postsynaptic membranes isolated from *Torpedo* electric tissue and the kinetics of AcCh-induced efflux of $^{22}Na^+$ from the vesicles. The binding studies define the rate constant, k_s, of the slow conformational transition resulting in the receptor conformation binding AcCh with high affinity [K_d = 2 nM, k_{dis} = 0.04 s^{-1} at 4 °C [Boyd, N. D., & Cohen, J. B. (1980) *Biochemistry 19*, 5344–5353, 5353–5358]]. For AcCh concentrations between 0.1 and 15 μM, k_s was characterized by a Hill coefficient of 1, $(k_s)_{max} \sim 0.8$ s^{-1}, and 10 μM AcCh produced a half-maximal rate. The observed $^{22}Na^+$ efflux, measured at times from 24 ms to 1000 s, defined the initial rate of $^{22}Na^+$ efflux (rate constant, k_o, a parameter proportional to the number of open channels) and a rate constant (k_d) for desensitization. Values of k_d were determined for AcCh concentrations between 0.1 and 1 μM where desensitization occurred before full release of $^{22}Na^+$ from the vesicles; for higher AcCh concentrations only k_o was determined. The observed concentration dependence of flux desensitization was the same as that of the conformational transition (k_s) defined by ligand binding, a result indicating that the high-affinity receptor is functionally desensitized. For AcCh concentrations to 300 μM, k_o was characterized by a Hill coefficient of 1.9 ± 0.1, $(k_o)_{max} \sim 420$ s^{-1}, and 150 μM AcCh produced a half-maximal response. These results establish that in the absence of agonist less than 1 channel in 10^7 is open and that the binding of two AcCh is necessary for channel activation. Comparison of the parameters characterizing [^{3}H]AcCh binding and channel activation indicates that the transient low-affinity conformation detected in binding studies reflects a desensitized receptor conformation that limits ion transport at subsecond times.

The binding of acetylcholine (AcCh)[1] by the nicotinic cholinergic receptor results within a fraction of a millisecond in a permeability response, the opening of a transmembrane ion channel. Binding of AcCh at equilibrium does not produce the response, since exposure to a constant concentration of AcCh for seconds or longer results in a reversible decline (desensitization) of the permeability response. Channel activation and desensitization have been interpreted by models in which the receptor (the AcCh binding protein and its ion channel) exists in distinct conformations differing both in ligand affinities and in the functional state of the ion channel [for reviews, see Colquhoun (1979) and Adams (1981)]. The simultaneous determination of the kinetics of binding of AcCh and of the functional state of the ion channel would provide direct evidence for the postulated receptor conformations.

Nicotinic postsynaptic membranes isolated from *Torpedo* electric tissue provide a unique preparation for the measurement of both the kinetics of ligand binding and the associated permeability response [for a review, see Karlin (1980)]. The effects of cholinergic agonists on the kinetics of binding of radiolabeled α-neurotoxins provided the first evidence of slow conformational transitions associated with the binding of agonists (Weber et al., 1975; Weiland et al., 1977). The results were generally compatible with a reaction model (eq 1) in



(1)

which the receptor contains a single class of AcCh binding sites that exists in the absence of ligand in two interconvertible conformations, R_c and R_d, binding agonist with low (K_c) and high (K_d) affinity, respectively. This model is similar to that proposed by Katz & Thesleff (1957) and Rang & Ritter (1970) to account for desensitization at the vertebrate neuromuscular junction, and it was reasonable to assume that R_d represented a functionally desensitized conformation, while binding to R_c was related to channel activation.

Further quantitative definition of receptor conformational equilibria has resulted from the introduction of rapid-mixing and ultrafiltration techniques to measure the kinetics of binding of [^{3}H]AcCh and [^{3}H]carbamylcholine (Boyd & Cohen, 1980a,b) and the use of stopped-flow fluorescence to analyze the binding of a fluorescent agonist (Heidmann & Changeux, 1979a,b, 1980). The general conclusions from both studies were similar and provided additional data compatible with the

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[1] Abbreviations: AcCh, acetylcholine; α-BgTx, α-bungarotoxin; Carb, carbamylcholine; TPS, *Torpedo* physiological saline (250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM sodium phosphate, pH 7).

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cyclic reaction mechanism. Agonist association kinetics were characterized by three kinetically distinct components: (1) The first was a rapid reaction associated with binding to 15% of the sites preexisting in a high-affinity conformation (R_d). (2) The second was an intermediate phase of the kinetics attributed to binding to 85% of the sites in a low-affinity conformation. The observed low-affinity binding probably reflected a more complex process in which ligand binding was coupled to a conformational transition. (3) The third was a slow phase of the reaction resulting in the final equilibrium binding in which greater than 99% of liganded receptors were in R_d.

Suspensions of *Torpedo* postsynaptic membranes contain vesicles sealed to $^{22}Na^+$, and the permeability response can be measured in terms of the agonist-induced flux of ions from these vesicles (Popot et al., 1976). With transport measured by manual filtration techniques, data can be obtained no sooner than 10 s after addition of agonist. The measured responses cannot be related directly to the number of open channels (Neubig & Cohen, 1980), but it was possible to demonstrate desensitization of the permeability response (Sugiyama et al., 1976; Bernhardt & Neumann, 1978). The use of rapid-mixing, quenched-flow methods (Hess et al., 1979, 1981; Cash & Hess, 1980; Neubig & Cohen, 1980) to measure ion transport on the time scale of milliseconds permits a quantitative definition of the rate of agonist-induced ion transport, a parameter proportional to the number of open channels. For carbamylcholine (Carb) the maximal rate constant for $^{22}Na^+$ flux represented an increase of about 10^6 over the unstimulated efflux rate, and the dose dependence of the stimulated efflux was characterized by a Hill coefficient of 2 for Carb concentrations between 3 μM and 1 mM (Neubig & Cohen, 1980). Comparison of the concentration dependence of Carb binding with that of channel activation indicated that observed transient low-affinity binding (Boyd & Cohen, 1980a) was not an "open channel" conformation.

In this report we describe a comparison of the binding of [^{3}H]AcCh by *Torpedo* membranes with ion flux responses generated in the identical system under identical conditions. Rapid-mixing, ultrafiltration techniques are used to define ligand binding kinetics for [^{3}H]AcCh concentrations up to 15 μM, while rapid-mixing, quenched-flow techniques are used to measure AcCh-induced $^{22}Na^+$ transport and the kinetics of desensitization of the flux response. The data are used to define acceptable models for activation and desensitization of the *Torpedo* nicotinic receptors by AcCh.

Materials and Methods

Preparation of Postsynaptic Membranes. Membranes were prepared from freshly dissected *Torpedo californica* and *Torpedo nobiliana* electric organs basically as described by Sobel et al. (1977). The membranes were stored at 4 °C in 37% (w/w) sucrose–0.02% NaN_3. Receptor site concentrations were determined by measuring the binding of ^{3}H-labeled α-neurotoxin from *Naja nigricollis* or *Bungarus multicinctus* (Weber & Changeux, 1974; Neubig & Cohen, 1979), and membrane protein concentrations were determined by the method of Lowry et al. (1951), with bovine serum albumin as a standard. Specific activities (in micromoles of α-neurotoxin binding sites per gram of protein) were 1–2.2 (six fish). Experiments were carried out at 4 °C with membrane suspensions in *Torpedo* physiological saline (TPS; 250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM sodium phosphate, pH 7.0) supplemented with 0.1 mM diisopropyl phosphorofluoridate to inhibit residual acetylcholinesterase.

Radiochemicals. [^{3}H]Acetylcholine, [^{3}H]propionyl-αBgTx, and $^{22}Na^+$ were obtained from New England Nuclear. ^{3}H-Labeled α-neurotoxin from *N. nigricollis* was a gift from Dr. A. Menez. The specific radioactivity of the [^{3}H]AcCh and α-neurotoxins was determined as described by Neubig & Cohen (1979).

Kinetics of Binding of [^{3}H]AcCh. The binding of [^{3}H]AcCh to *Torpedo* membranes was determined by filtration on glass-fiber filters (Whatman GF/F). Manual mixing and filtration techniques were used as described (Boyd & Cohen, 1980a) to measure ligand binding for reaction times greater than 5 s. For reaction times between 0.1 and 10 s, an automated, rapid-mixing ultrafiltration apparatus (Boyd & Cohen, 1980b) was used, and the reaction was terminated by use of air pressure (27 psi) to force the reaction mixture from an aging tube through the filter. The apparatus was modified to include a fluid reservoir so that the filtered material could be subjected immediately to a wash solution, which normally consisted of 4 mL of TPS containing a 10-fold excess of nonradioactive AcCh. This wash would reduce the free [^{3}H]AcCh in the filters, and also it would prevent rebinding to the receptors of any [^{3}H]AcCh that dissociated during the time of the wash. [^{3}H]AcCh bound to the high-affinity receptor conformation dissociates with a half-time of 15 s at 4 °C (Boyd & Cohen, 1980a), and a brief wash pulse would allow [^{3}H]AcCh to dissociate from low-affinity conformations but not from that high-affinity conformation.

$^{22}Na^+$ Efflux. For all experiments, conditions to measure $^{22}Na^+$ efflux were established as described (Neubig & Cohen, 1980). Briefly, a concentrated membrane suspension in TPS was incubated overnight with $^{22}Na^+$ (40 μCi/mL). The suspension was passed over a Dowex 50-W ion-exchange column and then diluted with TPS. Diluted suspensions were allowed to equilibrate for 20 min before characterization of AcCh-stimulated efflux. During that time, $^{22}Na^+$ is released from poorly sealed vesicles, and in the absence of agonist $^{22}Na^+$ efflux from the remaining well-sealed vesicles is characterized by a rate constant of $\sim 1 \times 10^{-4}$ s^{-1}. $^{22}Na^+$ retention in the vesicles was determined by filtration on glass-fiber (Whatman GF/F) filters.

AcCh-stimulated $^{22}Na^+$ efflux was measured by three methods: (1) quenched-flow techniques for times between 25 and 500 ms after addition of agonist (Neubig & Cohen, 1980); (2) pulsed, quenched-flow techniques for times from 1 to 30 s (Neubig, 1980); (3) manual mixing and filtration for times greater than 10 s. The pulsed, quenched-flow apparatus was a modification of the four-syringe, quenched-flow apparatus. Activation of a pneumatic ram results in the mixing of a membrane suspension containing $^{22}Na^+$ with a TPS solution containing agonist. That mixture flows into an aging tube where it resides for a preset time (1–30 s). A second pneumatic ram is then activated, forcing the reaction mixture into a second mixer in which it is mixed with a quench solution containing 2 mM of tubocurarine and 0.2 mM meproadifen. $^{22}Na^+$ retained within the vesicles was then determined by manual filtration. The kinetics of $^{22}Na^+$ efflux observed in the presence of AcCh were analyzed as described (Neubig & Cohen, 1980; Neubig, 1980) in terms of two parameters: the rate constant characterizing the initial rate of $^{22}Na^+$ efflux, k_0, and the rate constant for desensitization of the response, k_d. For $^{22}Na^+$ efflux measured at millisecond times, k_o was determined by fitting the data to a single exponential. For low AcCh concentrations resulting in $^{22}Na^+$ efflux on a time scale of seconds, the kinetic parameters were determined by fitting[2] the data to the equation

$$F_t = F_{max}\{1 - \exp[-(k_o/k_d)[1 - \exp(-k_d t)]]\} \quad (2)$$

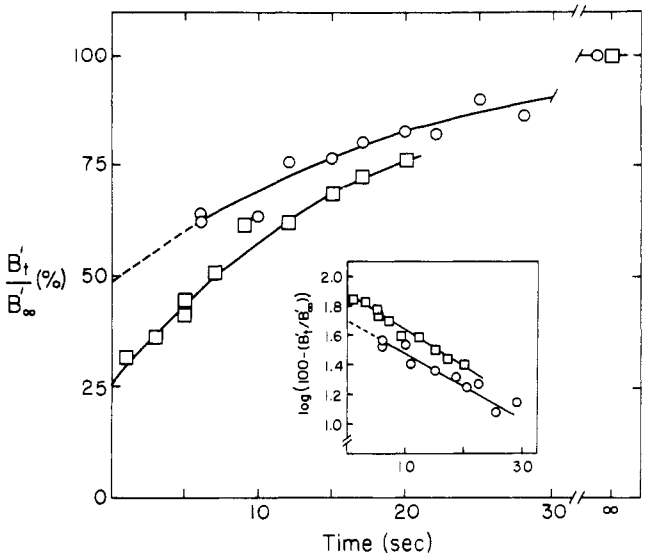

FIGURE 1: Kinetics of [^{3}H]AcCh binding to *Torpedo* membranes (4 °C). To measure binding without a filter wash, we mixed equal volumes of TPS containing 1800 nM [^{3}H]AcCh and 500 nM α-BgTx binding sites in a manual mixing apparatus and then filtered 1-mL aliquots through 1.3-cm (diameter) glass-fiber filters at the indicated times. Alternatively, membranes and [^{3}H]AcCh (0.65 mL) were mixed and filtered with an automated filtration apparatus with a 4-mL wash solution containing 20 μM AcCh in TPS. [^{3}H]AcCh bound specifically to the receptor is plotted as a percentage of the equilibrium binding for the wash (□) and no-wash (○) procedures. At equilibrium in the absence of a filter wash, the amount of [^{3}H]AcCh bound was 240 nM. After the wash, 49% (120 nM [^{3}H]AcCh) of the specifically bound [^{3}H]AcCh was retained on the filters, a result that indicates the amount of high-affinity binding that dissociates during the wash. (Inset) Semilogarithmic plots of [^{3}H]AcCh binding without (○) and with (□) wash.

F_t is the fraction of $^{22}Na^+$ released by time t and F_{max} is the maximal fraction of $^{22}Na^+$ released by high agonist concentrations. For each AcCh concentration a nonlinear least-squares computer program (Bard, 1967) was used to determine k_o and k_d.

Results

Kinetics of Binding of [^{3}H]AcCh. Analysis of the kinetics of binding of [^{3}H]AcCh to *Torpedo* membranes has been described (Boyd & Cohen, 1980a) for AcCh concentrations between 5 and 800 nM. In that study, definition of the rate constant (k_s) of the slow conformational transition was not possible for higher concentrations of AcCh for two reasons. First, with increasing AcCh concentrations the reaction amplitude reflecting the slow conformational transition became progressively smaller as most of the binding reaction was associated with the more rapid binding steps. Second, since no wash was used to remove free [^{3}H]AcCh from the filters, at higher AcCh concentrations, the [^{3}H]AcCh bound specifically to the receptor constituted too small a fraction of the total binding to permit precise measure.

To determine reaction rates for the slow phase of the association kinetics at higher [^{3}H]AcCh concentrations, we modified the filtration assay to include a filter wash. In Figure 1 the results of an experiment are shown in which the slow-phase binding kinetics were measured with and without the wash for a reaction mixture containing 900 nM [^{3}H]AcCh and 250 nM sites. The [^{3}H]AcCh bound specifically to the receptor (B_t') is expressed as a percent of the equilibrium binding (B_∞'). In the absence of any wash, B_t' represents the total amount of [^{3}H]AcCh bound specifically to all receptor conformations. When the data were analyzed in terms of a plot of log ($B_\infty' - B_t'$) against time (Figure 1, inset), the slow kinetics were linear and characterized by a rate consant, $k_s = (5.2 \pm 0.7) \times 10^{-2}$ s^{-1}. Extrapolation of the semilogarithmic plot to zero time determines the amount of binding occurring during the faster reaction phases. In the absence of any wash, 50% of the reaction amplitude was associated with the more rapid phases, an amplitude that includes binding to the preexisting high-affinity site (R_d, 22% of the sites for this preparation) as well as the transient low-affinity binding conformation (R_c). When the binding kinetics were measured with a filter wash, the observed slow-phase kinetics were also linear in the semilogarithmic plot, and the rate constant for the slow reaction [$k_s = (5.8 \pm 0.4) \times 10^{-2}$ s^{-1}] was the same as that determined in the absence of the wash. The amplitude of the reaction occurring rapidly (25% of the total reaction amplitude) was close to that expected if [^{3}H]AcCh bound to the low-affinity conformations dissociated during the wash. These results indicate that the filter wash can be used to determine the rate constants for the kinetics of formation of occupied, high-affinity receptors.

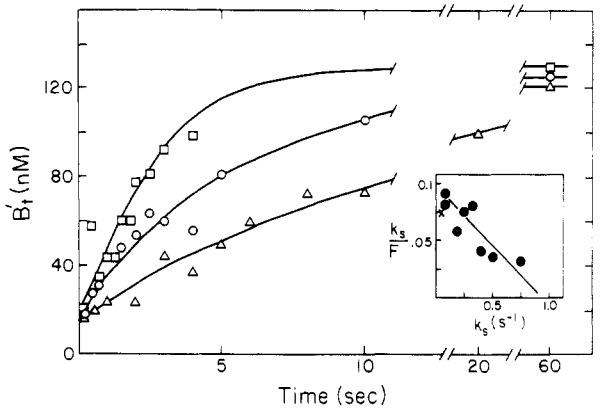

FIGURE 2: Kinetics of [^{3}H]AcCh binding to *Torpedo* membranes: slow-phase association kinetics. Membrane suspensions (500 nM α-BgTx binding sites) were mixed with [^{3}H]AcCh [2 (△), 6 (○), and 20 μM (□)] and filtered in an automated filtration apparatus utilizing a wash containing 500 μM AcCh. Specific binding of [^{3}H]AcCh is plotted against reaction time. The solid lines are best-fitting exponentials determined by least-squares analysis of semilogarithmic plots of the data. The rate constants (k_s) for the observed slow association kinetics were 0.08 (△), 0.17 (○), and 0.41 (□) s^{-1}. For each concentration about 15% of the observed reaction occurred rapidly, and ~50% of [^{3}H]AcCh bound to the high-affinity receptor dissociates during the wash (see Figure 1). (Inset) Eadie–Scatchard plot of dependence of k_s upon AcCh concentration with pooled data from three independent experiments and a value (×) from Boyd & Cohen (1980a).

For concentrations of [^{3}H]AcCh higher than 1 μM, the slow-phase reaction kinetics were measured by the use of the rapid-mixing ultrafiltration apparatus in conjunction with the filter wash. Results of a typical experiment are presented for [^{3}H]AcCh concentrations of 1, 3, and 10 μM (Figure 2). When analyzed in terms of plots of log ($B_\infty' - B_t'$) against time, the kinetics were linear and characterized by rate constants (k_s) of 0.082, 0.17, and 0.41 s^{-1}. The dependence of k_s upon AcCh concentration for this and similar experiments is presented in Figure 2 (inset) in an Eadie–Scatchard plot (k_s/[AcCh] against k_s). From such a plot, $(k_s)_{max} \sim 1$ s^{-1} and 10 μM AcCh results in a half-maximal value of k_s. The results of these and previous experiments provide a definition of k_s for AcCh concentrations varying from 10 nM to 10 μM. A

[2] This analysis assumes that desensitization is complete at long times. If this assumption is not valid, the values of k_o will be slightly overestimated while those for k_d slightly underestimated.

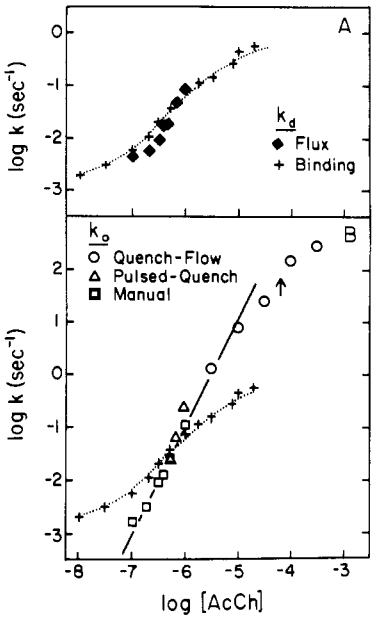

FIGURE 3: Rate constants characterizing channel activation and desensitization of *Torpedo* postsynaptic membranes by AcCh. (A) Desensitization rate constants, k_d, determined from $^{22}Na^+$ efflux experiments [(♦); see Materials and Methods] are compared with the rate constant (k_s) for the slow conformational transition to high-affinity receptor measured in [^{3}H]AcCh binding studies [(+); data from Boyd & Cohen (1980a) and this paper]. The dotted line is the value of k_s calculated for eq 1 with $K_c = 10$ μM, $k_2 = 0.7$ s^{-1}, and other parameters from Boyd & Cohen (1980a). (B) $^{22}Na^+$ efflux rate constants (k_o) determined from Figures 4 and 5 and similar experiments are shown by the open symbols [(□) manual method; (△) pulse quench; (○) quenched flow]. The solid line drawn through subsaturating responses has a slope of 2.0 and is compared with the concentration dependence of k_s (+).

plot of log k_s against log [AcCh] is presented in conjunction with data from the $^{22}Na^+$ efflux experiments (Figure 3).

AcCh-Stimulated $^{22}Na^+$ Efflux. AcCh concentrations less than 1 μM cause $^{22}Na^+$ release with a time course that was readily measurable by manual techniques (Figure 4). For 1 μM AcCh, however, about 75% of the $^{22}Na^+$ was released by the first time point (10 s). With the pulsed, quenched-flow apparatus over two-thirds of the efflux reaction could be measured for that concentration (Figure 4). Data from experiments such as these were analyzed according to eq 2 to determine k_o, the rate constant characterizing the initial rate of $^{22}Na^+$ efflux, and k_d, the rate constant for desensitization of the response. The solid lines in Figure 4 represent the nonlinear least-squares fit of the experimental data, and it can be seen that the data are fit quite well. For low concentrations of AcCh, k_d was similar in magnitude to k_o, so even at long times not all the $^{22}Na^+$ was released before full desensitization of the response. For example, for 0.2 μM AcCh where k_o was equal to $(3.8 \pm 0.7) \times 10^{-3}$ s^{-1} and k_o/k_d was equal to 0.39, only 40% of the $^{22}Na^+$ was released before full desensitization. Values of k_d could be determined from the efflux kinetics for AcCh concentrations between 0.1 and 1 μM, and over that range the rate constant for desensitization was within a factor of 2 of k_s, the rate constant for the slow conformational transition detected by binding studies (Figure 3A).

There was no evidence that a maximum $^{22}Na^+$ efflux rate was reached in the presence of 1 μM AcCh, and efflux of $^{22}Na^+$ in the presence of higher AcCh concentrations was measured by the quenched-flow technique (Figure 5). For AcCh concentrations of 3 and 10 μM, the observed efflux at subsecond times was reasonably well fit by single exponentials, with rate constants of 1.3 and 8 s^{-1} (solid curves in Figure 5A).

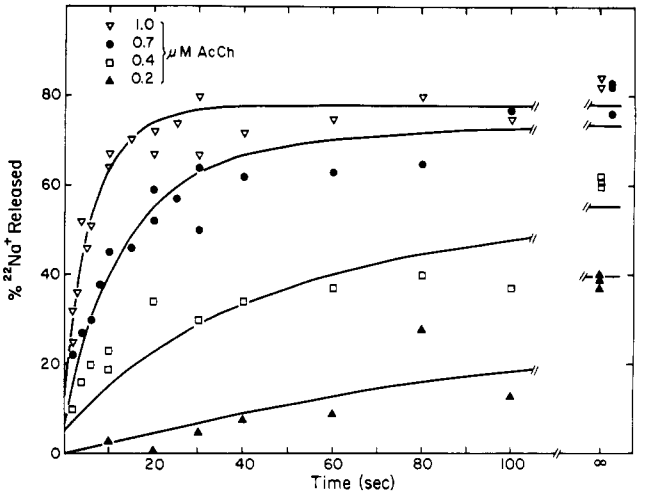

FIGURE 4: Kinetics of AcCh-stimulated $^{22}Na^+$ release from *Torpedo* vesicles at 4 °C. Manual mixing and filtration techniques were used to determine the amount of $^{22}Na^+$ released at times of 10–160 s after addition of AcCh. For times from 2 to 30 s, a pulsed-quench apparatus was utilized to mix $^{22}Na^+$-loaded membranes with AcCh and then to add at the indicated times an antagonist quench solution (final concentration 1 mM tubocurarine and 0.1 mM meproadifen). The quench solution stops the agonist-stimulated efflux, and $^{22}Na^+$ retained in the vesicles was determined by filtration. AcCh concentrations were 0.2 (▲), 0.4 (□), 0.7 (●), and 1 μM (▽). The data include experiments on three membrane preparations, so the amount of $^{22}Na^+$ released is expressed as a percentage of that released by 0.1 mM AcCh. The solid lines represent nonlinear least-squares fits of the data to the function (eq 2) including both activation and desensitization (see the text). Data for 120–160 s (omitted from the figure for clarity) and for 10–20 min were included in the analysis.

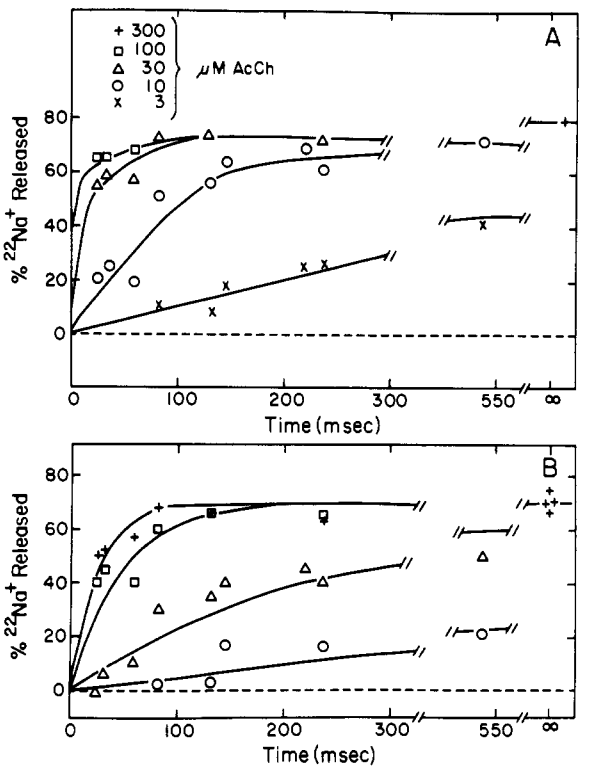

FIGURE 5: Quenched-flow determination of AcCh-induced efflux of $^{22}Na^+$ from *Torpedo* vesicles (4 °C). $^{22}Na^+$ released at times of 24–550 ms after addition of AcCh [3 (×), 10 (○), 30 (△) 100 (□), and 300 μM (+)] was measured for membrane suspension: (A) in the absence of α-BgTx and (B) after pretreatment with α-BgTx resulting in a blockade of 62% of the receptor sites.

For 30 μM AcCh or greater, most of the $^{22}Na^+$ was released by 24 ms, so initial rates of efflux could not be measured directly.

To obtain estimates of $^{22}Na^+$ efflux rates at these high AcCh concentrations, we measured $^{22}Na^+$ efflux from the same membrane suspension before and after blocking a fraction of the receptor sites with α-BgTx (Figure 5B). Occupancy of 62% of the sites by α-BgTx reduced the rate of $^{22}Na^+$ efflux at 10 μM AcCh by a factor of 7, and following this treatment $^{22}Na^+$ efflux at higher agonist concentration occurred over experimentally accessible times. For 30 μM AcCh, there was little release of $^{22}Na^+$ at the first measurable time, and full efflux kinetics were reasonably well-defined. In an initial analysis, the data were fit without weighting to a single exponential by linear least squares, and a rate constant of 3.4 ± 0.7 s^{-1} was obtained. For that fit, the calculated intercept at zero time was 13% $^{22}Na^+$ released. When the data were analyzed with a constraint of zero $^{22}Na^+$ release at zero time, a similar value of k_o (4 s^{-1}) was obtained, and the solid curve in Figure 5B is calculated for that k_o. For 100 and 300 μM AcCh, greater than half of the $^{22}Na^+$ was released by the first time point, and the solid curves in Figure 5B were calculated for the rate constants of 22 and 39 s^{-1} obtained when the fit was constrained to zero release at zero time. That constraint is physically reasonable, and the rate constants so obtained were larger than the rate constants of 10 and 9 s^{-1} obtained in the absence of any constraint. The rate constants for $^{22}Na^+$ efflux in the absence of α-BgTx were calculated from the rates observed in the presence of α-BgTx, since α-BgTx reduces the rate of $^{22}Na^+$ efflux by the same extent for different agonist concentrations (Neubig & Cohen, 1980).

The efflux rate constant, k_o, was determined for AcCh concentrations over more than 3 orders of magnitude by using the combination of manual methods and the quenched-flow technique. A log–log plot of the dependence of k_o on the concentration of AcCh is shown (Figure 3B, open symbols). This plot is linear for concentrations of AcCh less than 10 μM and has a slope 1.9 ± 0.1. This slope is equal to the Hill coefficient of the response, since for values of $k_o \ll k_{max}$, log k_o differs from log $[k_o/(k_{max} - k_o)]$ only by an additive term (i.e., $-\log k_{max}$). Thus the Hill coefficient can be determined without reference to a maximum response. It can be seen, though, from these plots that the response, k_o, does saturate at high agonist concentrations. In order to estimate the maximal response, we analyzed the data in a plot of $k_o^{-1/2}$ against 1/[AcCh]. This linearization has been used by others (Adams, 1975b; Cash & Hess, 1980) and is based on the Hill equation (1913):

$$R = R_{max}[A/(A + K_A)]^n \quad (3)$$

where R and R_{max} are responses, A is the agonist concentration, and K_A is an apparent dissociation constant for activation. The parameters obtained for $n = 2$ were $K_A = 70$ μM and $k_{max} = 420$ s^{-1} for AcCh.[3] The value of k_{max} for AcCh represents a stimulation of 3×10^6 over the resting efflux rate of 1.3×10^{-4} s^{-1}.

Discussion

We have used rapid-mixing techniques to measure under identical conditions the kinetics of binding of [^{3}H]AcCh to *Torpedo* membranes and the kinetics of the agonist-induced efflux of $^{22}Na^+$ from the vesicles. We used an automated ultrafiltration apparatus to characterize the rate constant (k_s) for the slow phase of the association reaction for AcCh concentrations up to 15 μM. Wash conditions were used to differentiate not only between free [^{3}H]AcCh and bound [^{3}H]AcCh but also between [^{3}H]AcCh bound to the high-affinity receptor conformation (K_d = 2 nM, k_{dis} = 0.03 s^{-1}) and that bound to transient low-affinity conformations from which [^{3}H]AcCh dissociates rapidly.

Binding Kinetics and Conformational Equilibria. The results obtained necessitate a revision of our conclusions (Boyd & Cohen, 1980a,b) concerning the appropriateness of the two conformation reaction cycle (eq 1) to account for all the binding data. In the previous studies both the observed concentration dependence of the amount of [^{3}H]AcCh binding occurring rapidly (B_f') and that of the rate constant (k_s) for the slow phase of the binding reaction were well accounted for by the two-conformation, cyclic reaction mechanism (eq 1). However, the apparent constant characterizing the transient low-affinity binding ($K_c \sim 1$ μM) was determined primarily by the concentration dependence of the binding occurring rapidly (B_f'), and with that value of K_c the limited concentration dependence of k_s was accounted for by $(k_s)_{max} \sim 0.06$ s^{-1}. As noted, a rigorous test of the reaction model depended upon experimental determination of the concentration dependence of k_s for AcCh concentrations above 1 μM. Our new results establish that $(k_s)_{max} \sim 1$ s^{-1} with a $C_{50} \sim 10$ μM (Figures 2 and 3). Because of the use of the filter–wash protocol, no new information has been obtained about the concentration dependence of the rapid phases of binding. Parameters can be found to reproduce the concentration dependence of k_s in terms of eq 1. The dotted lines in Figure 3 are calculated for K_c = 10 μM, a rate constant for $DR_c \rightleftharpoons DR_d$ of 0.7 s^{-1}, and the previously reported parameters (Boyd & Cohen, 1980a) for AcCh binding to R_d and for the unliganded conformational transition $R_c \rightleftharpoons R_d$. However, the new parameters do not account for the transient low-affinity binding ($C_{50} \sim 1$ μM) since they predict a C_{50} of 10 μM. Rather than focus solely upon an analysis of the binding data, we will consider the results of binding studies in conjunction with the observed concentration dependence of channel activation and flux desensitization.

Binding Kinetics and Flux Desensitization. We used rapid-mixing and quenched-flow techniques to measure the kinetics of efflux of $^{22}Na^+$ from *Torpedo* vesicles in the presence of AcCh concentrations varying from 0.1 to 300 μM. At the low AcCh concentrations where $^{22}Na^+$ efflux is measured on the time scale of seconds, the observed responses have been dissected to provide a definition of a rate constant (k_o) defining the rate of $^{22}Na^+$ efflux from the vesicles and a rate constant (k_d) characterizing the desensitization of the response. For the range of AcCh concentrations for which k_d could be determined, it was within a factor of 2 of k_s, the rate constant for the slow conformational transition of the *Torpedo* receptor detected by binding studies (Figure 3A). Thus the appearance of the liganded high-affinity receptor conformation has been correlated here with a functional measure of desensitization [see also Sine & Taylor (1979)]. Since the concentration dependence of k_s is well characterized by a Hill coefficient of 1, it is possible that a receptor binding only a single AcCh molecule undergoes the conformational transition resulting in desensitization.

$^{22}Na^+$ Efflux: Channel Activation. The parameter k_o represents a response directly proportional to the number of activated nicotinic receptors, and it has been possible to measure responses ranging over 5 orders of magnitude. For

[3] Parameters for Carb-stimulated $^{22}Na^+$ efflux are K_A = 780 μM and k_{max} = 190 s^{-1} when our previous efflux data (Neubig & Cohen, 1980) are analyzed in terms of eq 3 with values of k_o estimated from a least-squares fit of the data including the constraint of zero $^{22}Na^+$ released at zero time.

AcCh concentrations below 10 μM, k_o was determined from the $^{22}Na^+$ efflux kinetics that were fully defined by the rapid-mixing techniques used. For higher concentrations of AcCh, k_o could be determined only after the number of functional receptors per vesicle was reduced by an α-BgTx blockade. In the presence or absence of α-BgTx, for those AcCh concentrations producing $^{22}Na^+$ efflux at times from 20 to 500 ms, the data were reasonably well accounted for by a single exponential function (Figure 5). For AcCh concentrations of 100 and 300 μM, even with 62% of receptor sites occupied by α-BgTx, only a fraction of $^{22}Na^+$ efflux curves could be defined. We estimated k_o from those data by introducing a reasonable constraint, namely, that zero $^{22}Na^+$ was released at zero time. In the absence of that constraint, for 100 and 300 μM AcCh, the estimated values of k_o were 2- and 4-fold smaller, respectively. This discrepancy would result if there is an inactivation of ion transport at times <20 ms, but it could also reflect simply the limited data available to characterize ion transport induced by high AcCh concentrations. For those AcCh concentrations where k_o was estimated only after receptor blockade, it is possible that the inactivation procedure affects the estimate. This can be examined by characterizing the agonist-induced transport of a poorly translocated ion such as $^{45}Ca^{2+}$ for which maximal transport rates can be measured in the absence and presence of α-BgTx (Jeng & Cohen, 1982).

The data presented here permit a definition of agonist–dose response relations based upon ion transport measured after 20-ms exposure to AcCh, and it is appropriate to compare this characterization of nicotinic receptor activation with results obtained by other techniques. The dependence of k_o upon AcCh concentrations had been characterized in terms of three parameters: n, the Hill coefficient of the response; K_A, an apparent constant for channel activation; k_{max}, the maximal value of k_o.

The value for n has been estimated from the observed concentration dependence of k_o without any reference to the maximal response. For AcCh ($n = 1.9 \pm 0.1$), as for Carb [$n = 1.96 \pm 0.06$ (Neubig & Cohen, 1980)], n was equal, within experimental error, to 2.0. In quenched-flow analysis of Rb^+ influx into *Electrophorus* vesicles, responses were measured over a 200-fold range of concentration of AcCh and Carb (Hess et al., 1981), and those responses are also characterized by $n = 2$. The Hill coefficient of 2 and the effect of α-BgTx on receptor activation (Neubig & Cohen, 1980; Sine & Taylor, 1980) are consistent with a requirement for binding of two AcCh for channel activation. In fact, the concentration dependence of k_o indicates that if the monoliganded receptor is active, it is not more than 0.03% as active as the biliganded form (Neubig, 1980).

The apparent constant for channel activation ($K_A = 70$ μM) and the value of k_{max} (420 s^{-1}) were estimated from the concentration dependence of k_o by the use of eq 3 with $n = 2$. The value of K_A for AcCh in *Torpedo* vesicles is similar to that characterizing the AcCh-induced influx of Rb^+ in *Electrophorus* vesicles [$K_A \sim 80$ μM, Hess et al. (1981)] and $^{22}Na^+$ into chick muscle cells in culture [$K_A \sim 40$–50 μM, Catterall (1975)]. Voltage-clamp techniques have been used to characterize the permeability response in intact cells, and for both *Electrophorus* electrocyte (Sheridan & Lester, 1977) and frog neuromuscular junction (Dionne et al., 1978) for AcCh, $K_A \sim 25$ μM.

The measured response, k_o, characterizes the rate of agonist-induced $^{22}Na^+$ efflux from the population of *Torpedo* vesicles. The estimated value of k_{max} (420 s^{-1}) for AcCh represents a flux rate 3×10^6 times greater than that in the absence of agonist, and we can conclude that in the absence of agonist less than 1 channel in 10^7 is active. If all the unstimulated efflux is not receptor mediated or k_{max} is underestimated, even fewer channels can be open in the absence of agonist. The value of k_{max} reflects not only the transport properties of a single activated receptor but also the number of receptors per vesicle and the size of the vesicle. Thus the k_{max} characteristic of the AcCh response in the *Torpedo* vesicles cannot be compared directly to the transport properties of single open channels or to values of k_{max} measured from isotope flux experiments in *Electrophorus* vesicles or intact cells. However, the value of k_{max} reported here is equivalent[4] to a steady-state, unidirectional ion transport of ~50–100 Na^+ ions (α-BgTx site)$^{-1}$ ms^{-1}. The calculated transport rate is only about 5% of the rate expected if all ion channels were opened simultaneously, since a single open channel in muscle cells transports 12 000 ions/ms at −0.1 V (Anderson & Stevens, 1973) or about 1500 ions (α-BgTx site)$^{-1}$ ms^{-1} at 0 V if the membrane potential contributes a factor of 4.2 (Hodgkin & Katz, 1949) and there are 2 α-BgTx sites/functional unit.

The transport rate per receptor calculated from k_{max} in our studies appears distinctly lower than the transport in excess of 1000 ions (α-BgTx site)$^{-1}$ (ms)$^{-1}$ reported for *Torpedo* vesicles by Moore & Raftery (1980). Consideration of the origin of the different numbers reveals difficulties inherent in an apparently simple calculation. In that study, the response was the quenching by thallous ions of the fluorescence of a dye incorporated within the vesicles. For high concentrations of Carb, rate constants were estimated after inactivation by histrionicotoxin, a noncompetitive antagonist, rather than α-BgTx. The reported value of k_{max} (~1200 s^{-1}) at 25 °C was ~3-fold higher than our value for AcCh at 4 °C, and the higher calculated transport rate results from that factor, a factor of 2 from assumptions concerning vesicle size, and another factor of 2 for bidirectional transport. The experiments were actually conducted in a buffer containing 18 mM $TlNO_3$–18 mM $NaNO_3$, and in view of the many experimental differences, the similarity in the experimental observable k_{max} is more striking than the different estimates of maximal transport rate.

Rapid mixing and quenched flow have been used to measure agonist-induced Rb^+ influx into *Electrophorus* vesicles (Hess et al., 1981), and a maximal transport rate was reported of 2400 ions (α-BgTx site)$^{-1}$ ms^{-1}. This higher transport rate exists despite the fact that k_{max} for AcCh for *Torpedo* (420 s^{-1}) is 30-fold higher than that for *Electrophorus* (15 s^{-1}). Because *Torpedo* and *Electrophorus* vesicles contain respectively 1 and 0.005 nmol of α-BgTx sites/mg of protein, the fluxes observed in *Electrophorus* vesicles are associated with 200-fold fewer receptors. Thus receptors in *Electrophorus* vesicles are associated with higher transport rates when transport is measured by similar techniques and at similar times as in our experiments. While the difference may reflect limitations in our estimates of k_{max}, it is important to note that *Torpedo* nicotinic receptor is characterized by different conformational equilibria than those of *Electrophorus*. The equilibrium binding affinities for agonists are known to be 2 orders of magnitude higher for *Torpedo* than for *Electro-*

[4] This value is calculated from k_{max} (420 s^{-1}), the internal volume of the vesicles (~1 μL/mg of protein), the concentration of α-BgTx binding sites (~1 μmol/mg of protein), and the Na^+ concentration (250 mM). A unidirectional transport rate of 100 Na^+ α-$BgTx^{-1}$ ms^{-1} is calculated from k_{max}, the Na^+ concentration, and knowledge of the vesicle diameter [0.3 μm (Jeng et al., 1981)] and the surface density of receptors [10 000/μm^2 (Cartaud et al., 1978; Klymkowsky & Stroud, 1979)].

phorus (Weber & Changeux, 1974), and the slow agonist-induced conformational transition characteristic of agonist binding to *Torpedo* does not exist for *Electrophorus* vesicles (Epstein et al., 1980). Thus, even though values of K_A for channel activation are similar, there must be differences in the conformational equilibria determining desensitization and equilibrium binding, and for *Torpedo* there might be a rapid phase of desensitization limiting ion transport at millisecond times. Evidence has recently been obtained for two phases of flux desensitization in *Torpedo* vesicles (Walker et al., 1981), although the rapid desensitization ($t_{1/2} \sim 0.3$ s for saturating agonist) is much slower than we could predict.

Relation between Binding and Functional State. The appearance of the high-affinity receptor conformation has been correlated here with a functional measure of desensitization. A transient receptor conformation binding agonist with low affinity has been detected in studies of the binding of [^{3}H]-AcCh (Boyd & Cohen, 1980a,b) and of a fluorescent agonist (Heidmann & Changeux, 1979a,b, 1980). The half-maximal flux response ($C_{50} = 170$ μM) occurs for AcCh concentrations 200-fold higher than the concentration necessary to occupy 50% of the transient low-affinity sites ($C_{50} = 1$ μM). We discussed previously (Neubig & Cohen, 1980) that this discrepancy cannot be accounted for by reaction models containing three conformations [R_c (closed channel), R_o (open channel), and R_d (desensitized)] in which the conformational transitions leading to R_d are separated kinetically from more rapid processes associated with R_c and R_o. We consider it unlikely that the discrepancy results from the fact that the ion transport is due to a small fraction of active receptors and the observed binding to inactive receptors, and there are two other explanations that should be considered. (1) The binding site where agonists cause activation might be distinct from, but on the same receptor as, the binding site responsible for both the transient low affinity and the equilibrium binding. The latter might be considered a desensitization site! (2) The transient low-affinity binding indicates the existence of a fourth receptor conformation (R_p), a nontransporting conformation that results in reduced ion transport at millisecond times (Neubig & Cohen, 1980).

There has been considerable discussion concerning the possibility of separate binding sites for agonists and antagonists [for example, Gibson (1976) and Bulger et al. (1977)], and Adams (1975a) proposed that desensitization may be due to agonist binding to a second class of sites. Previous work (Neubig & Cohen, 1979) showed that the binding of agonists and antagonists was compatible with competitive inhibition. However, these studies would not have detected the postulated low-affinity activation sites ($K_D \sim 70$ μM for AcCh). Perhaps the best evidence against separate activation and high-affinity binding sites is the fact that bromoacetylcholine is an irreversible activator (Cox et al., 1979). All evidence suggests that this ligand binds irreversibly to the high-affinity agonist sites (Damle & Karlin, 1978; Wolosin et al., 1980), so presumably it also activates at this site. In the absence of any binding data demonstrating such a low-affinity site, it seems most likely that the site now known as the AcCh binding site is the activation site.

The ability to measure both AcCh binding and receptor activation under identical conditions has allowed the identification of the binding processes associated with desensitization of the nicotinic receptor. Efficient channel activation occurs for high concentrations of AcCh that result in fully occupied (biliganded) receptors. For low AcCh concentrations, desensitization occurs without channel activation as a result of binding to preexisting, desensitized (R_d) receptors; even for intermediate AcCh concentrations most receptors, which will be only singly occupied, desensitize without activation. This complex regulatory mechanism limits the maximal permeability response to those high AcCh concentrations characteristic of the early moments of quantal release. Further studies are necessary to delineate the mechanism of desensitization occurring at subsecond times.

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References

Adams, P. R. (1975a) *Pfluegers Arch. 360*, 135–144.
Adams, P. R. (1975b) *Pfluegers Arch. 360*, 155–164.
Adams, P. R. (1981) *J. Membr. Biol.* 58, 161–174.
Anderson, C. R., & Stevens, C. F. (1973) *J. Physiol. (London) 235*, 655–691.
Bard, Y. (1967) *Nonlinear Parameter Estimation and Programming*, Program 360D 13.6.003, IBM, Hawthorne, NY.
Bernhardt, J., & Neumann, E. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3756–3760.
Boyd, N. D., & Cohen, J. B. (1980a) *Biochemistry 19*, 5344–5353.
Boyd, N. D., & Cohen, J. B. (1980b) *Biochemistry 19*, 5353–5358.
Bulger, J. E., Fu, J.-J. L., Hindy, E. F., Silberstein, R. L., & Hess, G. P. (1977) *Biochemistry 16*, 684–692.
Cartaud, J., Benedetti, E., Sobel, A., & Changeux, J. P. (1978) *J. Cell Sci. 29*, 313–337.
Cash, D. J., & Hess, G. P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 842–846.
Catterall, W. A. (1975) *J. Biol. Chem. 250*, 1776–1781.
Colquhoun, D. (1979) in *The Receptors* (O'Brien, R. D., Ed.) Vol. 1, pp 93–142, Plenum Press, New York.
Cox, R. N., Karlin, A., & Brandt, P. M. (1979) *J. Membr. Biol. 51*, 133–144.
Damle, V. N., & Karlin, A. (1978) *Biochemistry 17*, 2039–2045.
Dionne, V. E., Steinbach, J. H., & Stevens, C. F. (1978) *J. Physiol. (London) 281*, 421–444.
Epstein, N., Hess, G. P., Kim, P. S., & Noble, R. L. (1980) *J. Membr. Biol. 56*, 133–137.
Gibson, R. E. (1976) *Biochemistry 15*, 3890–3901.
Heidmann, T., & Changeux, J. P. (1979a) *Eur. J. Biochem. 94*, 255–279.
Heidmann, T., & Changeux, J. P. (1979b) *Eur. J. Biochem. 94*, 281–296.
Heidmann, T., & Changeux, J. P. (1980) *Biochem. Biophys. Res. Commun. 97*, 889–896.
Hess, G. P., Cash, D. J., & Aoshima, H. (1979) *Nature (London) 282*, 329–331.
Hess, G. P., Aoshima, H., Cash, D. J., & Lenchitz, B. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1361–1365.
Hill, A. V. (1913) *Biochem. J. 7*, 471–480.
Hodgkin, A. L., & Katz, B. (1949) *J. Physiol. (London) 108*, 37–77.
Jeng, A. Y., & Cohen, J. B. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 1707A.
Jeng, A. Y., St. John, P. A., & Cohen, J. B. (1981) *Biochim. Biophys. Acta 646*, 411–421.
Karlin, A. (1980) *Cell Surf. Rev. 6*, 191–260.
Katz, B., & Thesleff, S. (1957) *J. Physiol. (London) 138*, 63–80.

Klymkowsky, M. W., & Stroud, R. M. (1979) *J. Mol. Biol. 128*, 319–331.
Lowry, O., Rosebrough, N., Farr, A., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Moore, H. P. H., & Raftery, M. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4509–4513.
Neubig, R. R. (1980) Ph.D. Thesis, Harvard University, Cambridge, MA.
Neubig, R. R., & Cohen, J. B. (1979) *Biochemistry 18*, 5464–5475.
Neubig, R. R., & Cohen, J. B. (1980) *Biochemistry 19*, 2770–2779.
Popot, J. L., Sugiyama, H., & Changeux, J. P. (1976) *J. Mol. Biol. 106*, 469–483.
Rang, H. P., & Ritter, J. M. (1970) *Mol. Pharmacol. 6*, 357–382.
Sheridan, R. E., & Lester, H. A. (1977) *J. Gen. Physiol. 70*, 187–219.
Sine, S., & Taylor, P. (1979) *J. Biol. Chem. 254*, 3315–3325.
Sine, S. M., & Taylor, P. (1980) *J. Biol. Chem. 255*, 10144–10156.
Sobel, A., Weber, M., & Changeux, J. P. (1977) *Eur. J. Biochem. 80*, 215–224.
Sugiyama, H., Popot, J.-L., & Changeux, J. P. (1976) *J. Mol. Biol. 106*, 485–496.
Walker, J., McNamee, M., Pasquale, E., Cash, D., & Hess, G. P. (1981) *Biochem. Biophys. Res. Commun. 100*, 86–90.
Weber, M., & Changeux, J. P. (1974) *Mol. Pharmacol. 10*, 1–40.
Weber, M., David-Pfeuty, T., & Changeux, J. P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3443–3447.
Weiland, G., Georgia, B., Lappi, S., Chignell, C. F., & Taylor, P. (1977) *J. Biol. Chem. 252*, 7648–7656.
Wolosin, J. M., Lyddiatt, A., Dolly, J. O., & Barnard, E. A. (1980) *Eur. J. Biochem. 109*, 495–505.

Lipid Environment of Acetylcholine Receptor from *Torpedo californica*†

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ABSTRACT: The lipid matrix of both acetylcholine receptor (AcChR)-rich and AcChR-poor membranes from *Torpedo californica* electroplax has been chemically characterized. AcChR-rich membranes contained higher levels of free cholesterol and slightly higher proportions of polyunsaturated ethanolamine phosphoglycerides. Major fatty acid components in the total lipid extracts from either membrane fraction were 24% palmitic, 20% docosahexaenoic, and 20% oleic acids. The fatty acid composition of individual lipid components from AcChR-rich and AcChR-poor membrane fractions was very similar. Native membranes and lipid vesicles, obtained by detergent dialysis from octyl glucoside solubilized lipid extracts, were used for the determination of the temperature-dependent rotational relaxation time of two fluorophores: diphenylhexatriene (DPH) and its trimethylammonium derivative (TMA-DPH). In both the DPH and the TMA-DPH domains, vesicles assembled with AcChR-rich membrane lipid exhibited a higher degree of rigidity than vesicles composed of AcChR-poor membrane lipid. The differences can be partially explained by their different cholesterol levels. As judged by Arrhenius plots, lipid phase separations or transitions were absent within the temperature range used in every native lipid system studied. In all cases, the presence of protein apparently induced more restraint on the rotational motion of the fluorophores. For example, higher rotational relaxation time values were obtained in AcChR-rich membranes than in corresponding plain lipid vesicles. This increase was less apparent with AcChR-poor membranes. Titration of the fluorescent pH probe 4-heptadecyl-7-hydroxycoumarin indicated that the apparent pK of the probe was influenced by the nature of the phospholipids and by the presence of protein. The pK value of coumarin was lower in vesicles formed with AcChR-rich membrane lipid (9.05) than in the intact native AcChR-rich membranes (9.25).

The nicotinic acetylcholine receptor (AcChR)[1] is an integral membrane glycoprotein mainly located at the neuromuscular synaptic junction and in the electric organ of certain elasmobranchs. Binding of specific agonists (acetylcholine) elicits translocation of ions which initiate postsynaptic depolarization. Purified AcChR as isolated from *Torpedo californica* has a molecular weight of 270 000 (Martinez-Carrion et al., 1975) and is composed of four different polypeptide chains (M_r 40 000, 50 000, 60 000, and 65 000) in an apparent 2:1:1:1 stoichiometry (Reynolds & Karlin, 1978; A. Paraschos, J. M. Gonzalez-Ros, and M. Martinez-Carrion, unpublished results).

The 40 000 molecular weight subunit is known to bear the binding site(s) for agonists, antagonists, and α-neurotoxins (Weill et al., 1974; Hsu & Raftery, 1979; Witzemann & Raftery, 1977, 1978; Lyddiatt et al., 1979; Damle et al., 1978) while the 50 000 and 60 000 molecular weight subunits appear to have large domains exposed to the membrane lipid environment (Sator et al., 1979; Gonzalez-Ros et al., 1980a). Few details, however, are available on the molecular mechanisms involved either in the formation of the "ion channel" initiating

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[1] Abbreviations: AcChR, acetylcholine receptor; OG, octyl β-D-glucopyranoside; α-Bgt, α-bungarotoxin; EP, ethanolamine phosphoglyceride; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; CARD, cardiolipin; PA, phosphatidic acid; SPH, sphingomyelin; l-PC, lysophosphatidylcholine; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid.

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