Ethanol Enhances Agonist-Induced Fast Desensitization in Nicotinic Acetylcholine Receptors†

Ge Wu and Keith W. Miller*

Department of Anesthesia, Massachusetts General Hospital, Boston, Massachusetts 02114, and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

Received January 31, 1994; Revised Manuscript Received May 25, 1994®

ABSTRACT: The reversible decline of the nicotinic acetylcholine receptor's response to acetylcholine during prolonged exposure to acetylcholine is known as desensitization. Here, we studied ethanol's modulation of fast agonist-induced desensitization of the nicotinic acetylcholine receptor in postsynaptic membrane vesicles from *Torpedo* using a fast kinetic technique: pulsed quenched flow. Preincubation of the vesicles with various concentrations of acetylcholine at 4 °C for times ranging from 80 ms to 1.5 s caused fast desensitization, which was revealed as a decreased $^{86}Rb^+$ influx when the vesicles were subsequently briefly exposed to a saturating concentration of acetylcholine in $^{86}RbCl$. Acetylcholine-induced fast desensitization had a maximum observed rate, k_d^{max}, of 6.8 s^{-1}, a half-effect concentration, K_D, of 157 μM, and a Hill coefficient of 1.4. Increasing the ethanol concentration up to 1.0 M causes a linear increase in k_d^{max}, such that 1.0 M ethanol doubles the rate. Ethanol (1 M) also decreased K_D 10-fold without changing the Hill coefficient. We consider a modified sequential model to interpret our data. Two acetylcholine molecules bind sequentially to the receptor's resting state to form a pre-open (closed) state, which then opens and, at very high acetylcholine concentrations, is inhibited. *A priori* fast desensitization might occur from any of these acetylcholine-occupied states. If we assume fast desensitization to occur solely from the pre-open state, our data predict an excessively large action of ethanol on the fast desensitization rate constant (>200-fold increase in the desensitization rate constant at 1 M ethanol). When we assume fast desensitization to occur from all states, ethanol is seen to have two actions. It shifts the equilibrium between the closed and open states toward the open state, accounting for the shift in the concentration response curve for fast desensitization, and it enhances the fast desensitization rate constant, accounting for the observed increase in the desensitization rate. As a consequence of this dual action, ethanol's net effect depends on both the agonist's concentration and its duration of action. Physiological concentrations of ethanol have their most pronounced effects at low agonist concentrations.

The binding of acetylcholine to the nicotinic acetylcholine receptor (nAcChoR)[1] in the postsynaptic membrane causes the opening of transmembrane ion channels within a fraction of millisecond, resulting in positively charged inorganic ion movement across the postsynaptic cell membranes and cell depolarization. However, prolonged exposure to acetylcholine results in a reversible decline of receptor response, known as desensitization, that was first demonstrated in frog muscle by electrophysiological methods (Katz & Thesleff, 1957). Subsequently, desensitization has been observed with several other members of this superfamily, such as the GABA (Epstein & Grundfest, 1970), glycine (Krishtal et al., 1988), and glutamate (Trussell & Fischbach, 1989) receptors. Agonist-induced desensitization is kinetically complex and occurs in two phases, both involving receptor conformational changes and increased affinity for agonists. The first occurs on the millisecond to second time scale (fast desensitization) and the second on the second to minute time scale (slow desensitization) [for reviews, see Changeux (1990), Changeux et al. (1984), and Udgaonkar and Hess (1986)].

Fast desensitization is an intrinsic property of the receptor, a process that normally occurs with a first-order rate constant of 2–7 s^{-1} (Feltz & Trautmann, 1982; Sakmann et al., 1980; Walker et al., 1982). No energy source nor ionic gradient is needed to drive the conformational change of the receptor between the resting and desensitized states. The recovery rate from the desensitized to the resting state is much slower than the forward rate (Aoshima et al., 1981; Dilger & Liu, 1992). Several factors, such as agonist concentration, membrane potential, temperature, calcium, and peptides, affect the fast desensitization rate [for a review, see Ochoa et al. (1989)], which is also accelerated by phosphorylation of the receptor (Huganir et al., 1986).

Ethanol has been shown to enhance nAcChoR-mediated cation flux at the neuromuscular junction in a number of electrophysiological studies (Bradley et al., 1980; Gage et al., 1975) without the nAcChoR channel conductance being altered (Nelson & Sachs, 1981). Both electrophysiological and rapid cation flux studies showed that ethanol acts by modulating the apparent affinity of cholinergic agonists for the channel activation process on the nAcChoR (Bradley et al., 1984; Dreyer et al., 1978; Forman et al., 1989; Linder et al., 1984), but these studies did not resolve whether ethanol changes the receptor's intrinsic affinity for agonist (K_1) or the closed–open state equilibrium (K_o). This uncertainty made it difficult to interpret the observation that ethanol (0.5 M), although it did not itself facilitate fast desensitization, did

† This research was supported by a grant from the National Institute on Alcohol Abuse and Alcoholism (AA-07040) to the Department of Anesthesia, Massachusetts General Hospital.

* Address for correspondence: Professor Keith W. Miller, Department of Anesthesia, Massachusetts General Hospital, Boston, Massachusetts 02114.

® Abstract published in *Advance ACS Abstracts,* July 1, 1994.

[1] Abbreviations: nAcChoR, nicotinic acetylcholine receptor; α-BTX, α-bungarotoxin; TPS, *Torpedo* physiological saline; DFP, diisopropylphosphorofluoridate.

0006-2960/94/0433-9085$04.50/0

enhance acetylcholine-induced fast desensitization (Forman et al., 1989). However, because the acetylcholine concentration response curves for initial flux rate and fast desensitization rate both have the same Hill coefficient and half-effect concentration, it seemed most parsimonious to assume that ethanol acted on the binding step, K_1 (Forman & Miller, 1988).

On the contrary, we recently have shown that ethanol stabilizes the open channel state by changing K_o rather than K_1 (Wu et al., 1994). In light of this new finding, we have undertaken a detailed reexamination of the ethanol concentration dependence of the enhancement of agonist-induced fast desensitization using the pulsed quenched-flow flux assay in *Torpedo* acetylcholine receptor-rich vesicles. Our data are most compatible with a model in which fast desensitization proceeds from all agonist-occupied transient states with an equal rate and ethanol acts both by displacing the closed–open equilibrium toward the open state and by increasing the intrinsic rate constant for fast desensitization.

MATERIALS AND METHODS

Diisopropyl phosphorofluoridate (DFP) was from Aldrich Chemical Co. (Milwaukee, WI). α-Bungarotoxin (α-BTX) was from Miami Serpentarium (Miami, FL) and was purified by CM-cellulose chromatography (Lee et al., 1972). Acetylcholine chloride, procaine hydrochloride, sucrose, buffer reagents, and Dowex resin were from Sigma Chemical Co. (St. Louis, MO). ^{86}RbCl was from New England Nuclear (Boston, MA).

Postsynaptic membranes from freshly dissected electroplaques of *Torpedo nobiliana* (Biofish Associates, Georgetown, MA) were prepared using sucrose density gradient centrifugation at 4 °C essentially as described previously (Braswell et al., 1984). Membrane suspensions were kept frozen at −80 °C in *Torpedo* physiological saline (TPS: 250 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM PO_4^{2-}, and 0.02% Na_3N, pH 7.0) and thawed within 48 h of use. Prior to flux assays, vesicles were incubated for 20 min with 0.1 mM DFP at 4 °C to inhibit acetylcholinesterase activity. Flux responses were not altered by this treatment (Forman et al., 1989).

Channel function was assayed by measuring agonist-induced $^{86}Rb^+$ influx into spontaneously sealed native *Torpedo* electroplaque vesicles at 4 °C. A rapid quenched-flow technique, as described in detail previously (Forman et al., 1987), was used for time-resolved studies. Three mixers were separated by different lengths of tubing to control the incubation time after mixing. At the first mixer, receptors were mixed with different concentrations of acetylcholine and ethanol when appropriate. At the second mixer, the solution from first mixer was mixed with a solution of $^{86}Rb^+$ and acetylcholine (final concentration, 1 mM), and influx was initiated for 8 ms. At the third mixer, the solution from the second mixer was mixed with procaine (final concentration, 50 mM) in order to quench $^{86}Rb^+$ influx. Preincubation times were calibrated by base hydrolysis of *o*-nitrophenyl acetate. These times agreed with those calculated from measurements of the flow rate through the rapid mixing device and the volume between the mixers. When necessary, the number of active receptor–channel complexes was reduced by blocking with the irreversible inhibitor, α-BTX, to prevent full equilibration of $^{86}Rb^+$ within the experimental assay time (Miller et al., 1987). Ethanol was added to vesicles at the same time as the agonist at the first mixer. Following quenching at the third mixer, the reaction mixture was *immediately* (in order to minimize the time of exposure to ethanol) transferred to a small cation exchange column (Dowex-50Wx8, 100–200 mesh, about 700-μL bed volume), and the vesicles were eluted with 700 μl (200 mM) of sucrose solution, then mixed with 4 mL of Packard Ultima Gold XR (Meriden, CT) scintillation solution and assayed in a Packard 1900CA liquid scintillation analyzer.

The percentage of non-leak $^{86}Rb^+$ counts inside the vesicles, F_A, is calculated from

$$F_A = \frac{\text{CPM(Ag,}t\text{)} - \text{CPM(leak,}t\text{)}}{\text{CPM(total)} - \text{CPM(leak,}t\text{)}} \quad (1)$$

where CPM(Ag,t) is the counts per minute of $^{86}Rb^+$ in the vesicles following exposure to acetylcholine in the quenched-flow apparatus and passage through the cation exchange column. CPM(leak,t) is the $^{86}Rb^+$ counts that leaked into the vesicles in the absence of agonist and is obtained by an experiment identical to that used for CPM(Ag,t), except that the acetylcholine concentration is zero in the buffer during the leak measurement. CPM(total) is the total $^{86}Rb^+$ in the vesicles at equilibrium, obtained by allowing $^{86}Rb^+$ to diffuse into the vesicles overnight and then applying them to the cation exchange column.

Initial $^{86}Rb^+$ influx rates (k_f) were estimated from eq 2, which corrects the response for the decline in the $^{86}Rb^+$ concentration gradient as the assay progresses:

$$F_c = -\ln(1 - F_A/F_{eq}) = k_f t \quad (2)$$

where F_{eq} is the maximum acetylcholine-induced flux response possible in this system. The value of F_{eq} was measured for each batch of vesicles by exposing them to 1 mM acetylcholine for 10 s in the absence of α-BTX and making the leak correction as in eq 1 with F_{eq} replacing F_A. Only at the highest levels of α-BTX block is F_{eq} reduced in the 10-s flux assay (Miller et al., 1991); we observed no change with the amount of α-BTX used here. The value of F_{eq} did not differ from one in the presence or absence of α-BTX.

The nAcChoR activity, F_c, after preincubation with acetylcholine is denoted as $F_{Ach,t}$, which is dependent on both the preincubation acetylcholine concentration (subscript Ach) and preincubation time (subscript t). $F_{Ach,t}$ was determined from the amount of $^{86}Rb^+$ that entered the sealed vesicles in 8 ms in the presence of 1 mM acetylcholine after preincubation with acetylcholine (that is, between mixer two and three). It has been shown (Forman & Miller, 1988) that $F_{Ach,t}$ can be described by the following equation:

$$\ln(F_{Ach,t}/F_{Ctrl}) = -k'_d t + C \quad (3)$$

where t is the preincubation time with acetylcholine and F_{Ctrl} was measured similarly to $F_{Ach,t}$, except that preincubation was with TPS instead of acetylcholine. The intercept, C, is a measure of the fraction of receptors that is inhibited at high concentrations of agonist. If the preincubation concentration of acetylcholine is below 5 mM, $C = 0$; if it is above 15 mM, $C \neq 0$ (Forman & Miller, 1988). The slope, k'_d, is a measure of the microscopic desensitization rates from states AR, A_2R, and A_2R^o (see Appendix). It is the apparent fast desensitization rate in the present experiments since the acetylcholine used here is always less than 15 mM. Thus, the plot of $\ln(F_{Ach,t}/F_{Ctrl})$ vs t is linear and passes through the origin in the present experiments ($C = 0$; see Figures 1 and 2).

The relationship between k'_d and the preincubation acetylcholine concentration can be described by the following Hill equation:

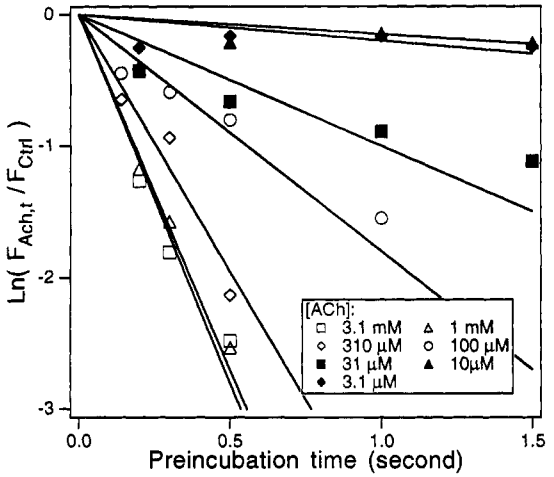


FIGURE 1: Concentration dependence of acetylcholine-induced fast desensitization in the absence of ethanol. $\ln(F_{Ach,t}/F_{Ctrl})$ vs the time of preincubation with different concentrations of acetylcholine is shown. $F_{Ach,t}$ is obtained by preincubating the nAcChoR vesicles with acetylcholine (3 μM to 3 mM) for time t and then mixing them with 1 mM acetylcholine and $^{86}Rb^+$ for 8 ms. F_{Ctrl} is obtained by measuring the influx $^{86}Rb^+$ into the same vesicles under conditions identical to those used in measuring $F_{Ach,t}$, but TPS is used instead of acetylcholine for preincubation. The solid lines are obtained by a linear least-squares fit. The slopes of the fitted lines are the apparent desensitization rates k'_d at the different acetylcholine concentrations.

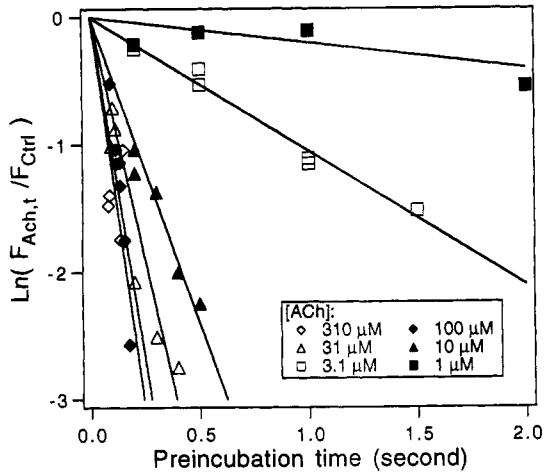


FIGURE 2: Concentration dependence of acetylcholine-induced fast desensitization in the presence of 1 M ethanol. $\ln(F_{Ach,t}/F_{Ctrl})$ vs the time of preincubation with different concentrations of acetylcholine is shown. $F_{Ach,t}$ is obtained by preincubating the nAcChoR vesicles with acetylcholine (1 μM to 3 mM, beyond 0.1 mM the desensitization rates are similar and only the data from 1 μM to 0.3 mM are shown for clarity) plus 1 M ethanol for time t and then mixing them with 1 mM acetylcholine and $^{86}Rb^+$ and allowing $^{86}Rb^+$ influx for 8 ms. F_{Ctrl} is obtained in the same way as for Figure 1 but with ethanol in TPS. The solid lines are obtained by a linear least-squares fit. The slopes of the fitted lines are the apparent desensitization rates k'_d at the different acetylcholine concentrations.

$$k'_d = k_d^{max}\frac{[A]^n}{[A]^n + K_D{}^n} \quad (4)$$

where k_d^{max} is the maximum value of k'_d, which occurs at high preincubation acetylcholine concentration, K_D is the acetylcholine concentration producing 50% of the maximum desensitization, and n is the Hill coefficient.

RESULTS

The flux rate of $^{86}Rb^+$ through vesicles upon exposure to agonist depends on both the concentration of agonist used and the concentration of the receptors available for activation. In the presence of sufficient amounts of α-BTX, the flux rate k_f defined in eq 2 is proportional to integrated flux, F_c. If the flux time is fixed (8 ms in the present experiment), F_c is a measure of the flux rate. It is known that 1 mM acetylcholine is sufficient to activate all of the available receptors and elicit maximum flux (Colquhoun & Ogden, 1988; Forman et al., 1987) in either the presence or absence of ethanol (Forman et al., 1989). Thus, in the presence of 1 mM or more (but less than 5 mM to prevent acetylcholine self-inhibition) acetylcholine, F_c is a measure of the concentration of receptor available for activation. In the present assay, a fixed concentration of 1 mM acetylcholine was used at the second mixer to induce influx, and up to 3 mM acetylcholine was used in the first mixer for inactivation. Thus, the final concentration of acetylcholine during the influx assay always produced maximal stimulation.

To describe the decrease in available receptors due to desensitization after exposure to a concentration of acetylcholine for a certain time, the function $\ln(F_{Ach,t}/F_{Ctrl})$ is used (see the Materials and Methods section). It has been shown (Forman et al., 1989; Hess et al., 1979), in agreement with the present experiment, that $\ln(F_{Ach,t}/F_{Ctrl})$ decreases linearly with preincubation time at a fixed desensitizing concentration of acetylcholine within experimental error. Thus, in the absence of ethanol, the plot of $\ln(F_{Ach,t}/F_{Ctrl})$ against the time t of preincubation at different acetylcholine concentrations is shown in Figure 1. The apparent fast desensitization rate constant k'_d is obtained as the slope of the plot (see eq 3). At acetylcholine concentrations less than 10 μM, no detectable desensitization occurs on a time scale of less than 1.5 s. Above 10 μM acetylcholine, fast desensitization is detectable, and the rate of desensitization increases with increasing preincubation acetylcholine concentration up to 1 mM acetylcholine. At and above 1 mM acetylcholine, most of the flux activity of the nAcChoR disappeared within 0.5 s of preincubation.

The plot of $\ln(F_{Ach,t}/F_{Ctrl})$ against preincubation time t in the presence of 0.5 or 1 M ethanol is also linear for all preincubation acetylcholine concentrations, but the slope is much steeper than that at the same acetylcholine concentration in the absence of ethanol. This is illustrated in Figure 2 for 1 M ethanol. Significant fast desensitization is now detected even at 3 μM acetylcholine, and it saturates at 0.1 mM acetylcholine. At and above 0.1 mM acetylcholine, most of the flux activity of the nAcChoR disappeared after 0.25 s of preincubation (data not shown in Figure 2 for clarity).

The acetylcholine concentration dependence of the apparent fast desensitization rate constant k'_d is shown in Figure 3 in the absence and presence of 0.5 and 1 M ethanol. The data have been fit to eq 4 by a nonlinear least-squares procedure (solid lines), and the corresponding parameters n, K_D, and k_d^{max} are summarized in Table 1. It can be seen that both the maximum fast desensitization rate (k_d^{max}) and the apparent affinity of agonist causing desensitization ($1/K_D$) increase with increasing ethanol concentration. The Hill coefficients, n, did not differ, within experimental error.

Above 1 mM acetylcholine, the apparent fast desensitization rate, k'_d, has its maximum value, k_d^{max}, in the absence or presence of ethanol (see Figure 3). We studied the effects of ethanol concentration on k_d^{max} in more detail by measuring the k'_d with the preincubating acetylcholine concentration fixed at 1 mM. The resulting maximum desensitization rates, k_d^{max}, are plotted against ethanol concentration in Figure 4, which shows a linear increase with ethanol concentration. At

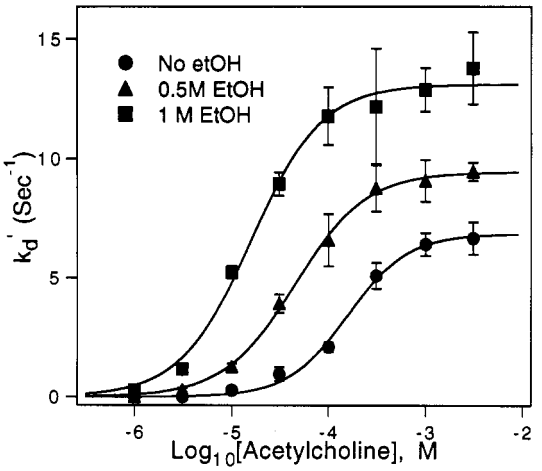


FIGURE 3: Dependence of the apparent desensitization rate, k'_d, on acetylcholine concentration in the absence (●) and presence of 0.5 (▲) and 1 M (■) ethanol. The solid lines were obtained by the nonlinear least-squares fit to the Hill equation (eq 4). The results of the fit are summarized in Table 1. Ethanol both increases the maximum desensitization rate and produces a leftward shift of the curves.

Table 1: Action of Ethanol on Acetylcholine-Induced Fast Desensitization of nAcChoR[a]

[EtOH] (M)	k_d^{max} (s^{-1})	n	K_D (μM)
0	6.8 ± 0.20	1.4 ± 0.2	157 ± 16
0.5	9.5 ± 0.16	1.2 ± 0.1	45 ● 2.9
1.0	13.1 ± 0.30	1.2 ± 0.1	15.8 ± 1.6

[a] Experimental parameters were obtained from fitting the data in Figure 3 to equation 4. The errors are obtained from the fit. With increasing ethanol up to 1 M, the apparent maximum desensitization rate doubled, the apparent affinity of acetylcholine for nAcChoR increased, and the Hill coefficient, n, barely changed.

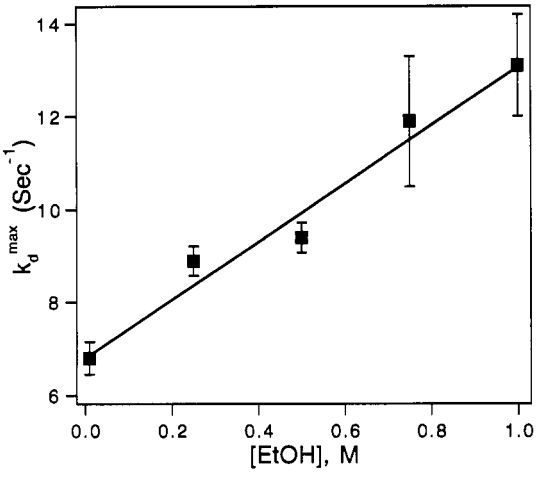
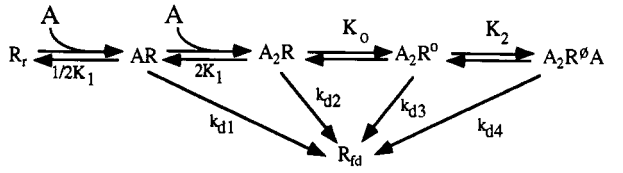


FIGURE 4: Apparent maximum desensitization rates (k_d^{max}) increasing linearly with increasing ethanol concentrations. k_d^{max} was obtained by preincubating nAcChoR vesicles with 1 mM acetylcholine and appropriate amounts of ethanol. The solid line was obtained by a linear least-squares fit to the data, yielding $y = (6.8 \pm 0.38) + (6.3 \pm 0.62)x$.

1.5 M ethanol, the maximum desensitization rate (14.0 ± 0.7 s^{-1}) appears not to have increased linearly. However, since ethanol increases cation leakage from the vesicles at such high concentrations, we confined our analysis to concentrations of 1 M or less. The solid line in Figure 4 is obtained from linear least-squares fit to the data up to 1 M ethanol, yielding an intercept of 6.8 ± 0.38 s^{-1} and a slope of 6.3 ± 0.62 s^{-1} M^{-1} ethanol.

Scheme 1



DISCUSSION

Kinetic Model. The nAcChoR *immediately* following mixing with acetylcholine can be described by a model that has five rapidly interconverting states coexisting at equilibrium (Scheme 1, top line). These are the resting state, R_r, the state with one agonist bound, AR, the state with two agonists bound, A_2R, the open channel state, A_2R^o, and the inhibited channel state, $A_2R^{\phi}A$ (Colquhoun & Ogden, 1988). After prolonged exposure to acetylcholine, agonist-induced fast-desensitized state(s) may be accessed from all four agonist-associated states (Scheme 1, lower line). In the present experiment, where the measured desensitization is observed as a monophasic decrease in the number of activatable receptors, no information about the details of the desensitized state(s) can be obtained. Therefore, we refer to the desensitized receptors collectively as R_{fd}, a procedure that has been adopted in a number of studies (Cachelin & Colquhoun, 1989; Forman et al., 1989).

In Scheme 1, k_{d1}, k_{d2}, k_{d3}, and k_{d4} are the first-order desensitization rate constants from AR, A_2R, A_2R^o, and $A_2R^{\phi}A$, respectively. The rates for reactivating the desensitized receptor are omitted because they are 10–1000-fold smaller than the corresponding forward rates (Aoshima et al., 1981; Dilger & Liu, 1992).

The following relationship between the slope in eq 3 and the individual microscopic rate constants can be obtained as a function of the agonist concentration (see Appendix):

$$-k'_d = \frac{k_{d1}2K_1K_o[A] + k_{d2}[A]^2K_o + k_{d3}[A]^2}{k_o(K_1 + [A])^2 + [A]^2} \quad (5)$$

Equation 5 shows that only desensitization from the AR, A_2R, and A_2R^o states contributes to k'_d no matter whether desensitization from the inhibited state ($A_2R^{\phi}A$) occurs (Dilger & Liu, 1992). This equation predicts that when $[A] \gg K_1$, k'_d becomes a constant independent of the acetylcholine concentration (Forman & Miller, 1988). If the fraction of $A_2R^{\phi}A$ is high ([A] > 15 mM), then a plot of $\ln(F_{Ach,t}/F_{Ctrl})$ against t will not pass through the origin (Forman & Miller, 1988).

Analysis. There is no unique solution for eq 5 because there are five parameters in it, but two stratagems make the situation tractable in practice. First, Scheme 1 may be simplified on the basis of previous work (Aoshima et al., 1981; Dilger & Liu, 1992). Second, we can restrict the range of values each parameter may take because some of the parameters (K_1, K_o) and their ethanol dependence have been estimated independently (Aoshima et al., 1981; Colquhoun & Ogden, 1988; Dilger & Liu, 1992; Sine et al., 1990; Wu et al., 1994). We will show here that by employing these constraints, conclusions about the role of ethanol in fast desensitization may be drawn with considerable certainty.

If we assume that the rate of fast desensitization from each of the three states in Scheme 1 is equal and has the value k_d, eq 5 yields

$$-k'_d = \frac{k_d(2K_1K_o[A] + [A]^2K_o + [A]^2)}{K_o(K_1 + [A])^2 + [A]^2} \quad (6)$$

For acetylcholine, K_o is small with a value of 0.03 at the frog end-plate (Colquhoun & Ogden, 1988). In the absence of calcium, K_o values of 0.68 at 22 °C and 0.1 at 12 °C are determined for *Torpedo* nAcChoR expressed in fibroblasts (Sine et al., 1990). Flux data cannot yield an absolute value of K_o. However, recent studies on nAcChoR identified K_o as the primary site of ethanol's action in the upper row of Scheme 1 (Wu et al., 1994; Dilger et al., 1994). K_o in our vesicles must be smaller than 0.05 or we would detect an ethanol-induced increase in maximum acetylcholine-induced flux. In fact, the initial flux rate data of Forman et al. (1989) can be fit to the two-site sequential binding model with $K_o = 0.039$ (Wu et al., 1994). Thus, the above assumption is effectively equivalent to assuming that most receptors desensitize from the open channel state since it accounts for most of the receptor population because of the small value of K_o.

Our recent study (Wu et al., 1994) also allowed us to estimate the dependence of K_o on ethanol concentration for acetylcholine (Figure 5A), and the values are given in Table 2A. Using them to fit our acetylcholine concentration–fast desensitization rate curves in the presence of ethanol, as shown in Figure 5B, they correctly predict the increase in the agonist's apparent affinity for this effect and show that the fast desensitization rate increases roughly 2-fold between 0 and 1 M ethanol while K_1 barely changes. The leftward shift of the acetylcholine concentration–flux response curves and the acetylcholine concentration k'_d response curves in the presence of ethanol can both be accounted for by the increase in K_o, as seen in Figure 5.

The above conclusion (Table 2A) depends on the parsimonious assumption that ethanol acts similarly on the initial flux rates of both suberyldicholine and acetylcholine. While this is most probably true, one might argue to the contrary that (i) suberyldicholine activates the channel with a Hill coefficient of 1, whereas acetylcholine does so with a Hill coefficient approaching 2, and (ii) the two agonists are of considerably different size, so that were ethanol to affect the agonist binding site over a region larger than that which interacts with acetylcholine, its action on the two agonists might not be identical. Although we feel this is unlikely, we cannot entirely rule it out and, therefore, here examine the other limiting case, which is that acetylcholine's K_o is equal to 0.039 and is unaffected by ethanol which instead acts solely on acetylcholine's K_1. This assumption produces a satisfactory fit of our data for fast desensitization, and the results are shown in Table 2B.

The important conclusion that emerges from the analysis in Table 2 is that k_d varies 2-fold as the concentration of ethanol varies from 0 to 1 M, independent of whether within the confines of Scheme 1 we assume that ethanol acts on K_o or K_1. That this conclusion concerning k_d is secure follows from the fact that it is the maximum in the acetylcholine concentration–fast desensitization curves in Figure 3 that defines the rate constant for fast desensitization; the shift in the observed K_D may be assigned either to K_o or K_1 without altering the conclusion regarding fast desensitization.

Finally, we turn to the question of whether fast desensitization occurs exclusively from the pre-open states, AR and A_2R, as it is convenient to assume in a minimalist scheme (Aoshima et al., 1981; Hess, 1993). Rapid concentration jump experiments first provided evidence that all of the states might contribute equally to fast desensitization (Forman & Miller, 1989). More recently, fast-flow patch clamp studies have confirmed this finding (Dilger & Liu, 1992), but even with this technique it remains impossible to entirely rule out that fast desensitization occurs exclusively from the pre-open states,

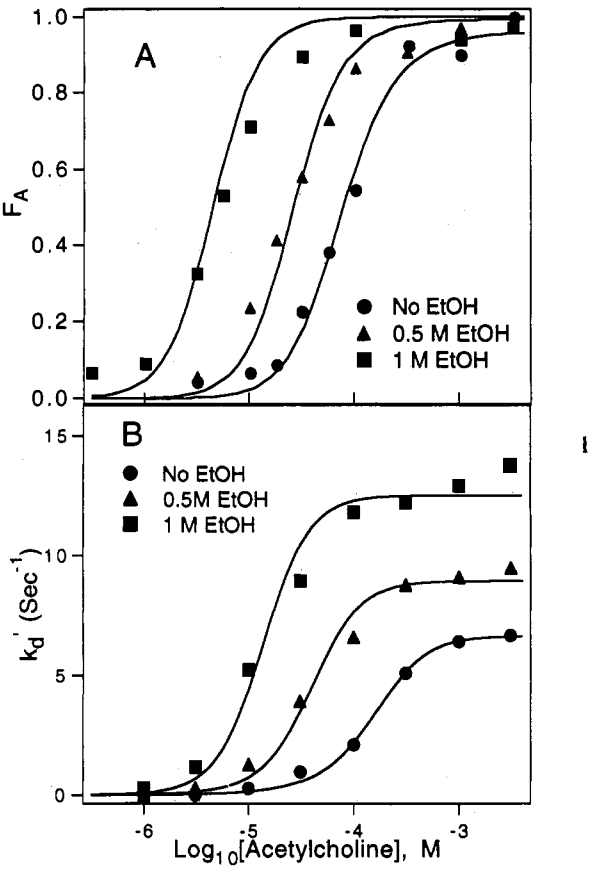


FIGURE 5: Test of the kinetic model in Scheme 1. Ethanol's observed effect on (A) the initial flux rate, F_A [data taken from Forman et al. (1989)], and (B) the rate of fast desensitization, k'_d, is shown by the filled data points (●, no ethanol; ▲, 0.5 M ethanol; and ■, 1.0 M ethanol). The solid lines are the theoretical curves obtained by a nonlinear least-squares fit to the model in Scheme 1, with the assumptions and parameters given in Table 2A.

Table 2: Results for a Theoretical Model Assuming Fast Desensitization Occurs at a Single Rate from All of the Occupied States[a]

[EtOH] (M)	assumed K_o	k_d (s^{-1})	K_1 (μM)
	A[b]		
0	0.039	6.7 ± 0.12	972 ± 53
0.5	0.0056	9.1 ± 0.30	591 ± 116
1.0	0.00028	12.7 ± 0.41	806 ± 93
	B[c]		
0	0.039	6.7 ● 0.12	972 ± 53
0.5	0.039	9.0 ● 0.27	262 ± 31
1.0	0.039	12.6 ± 0.36	93 ± 12

[a] Parameters were obtained from fitting the data in Figure 3 to equation 6. The errors are obtained from the fit. [b] We assume that K_o varies with ethanol concentration in the manner obtained previously by Wu et al. (1994) and shown in the table. As ethanol increases from 0 to 1.0 M, the microscopic fast desensitization rate (k_d) increases by about the same amount as the observed k_d^{max} (see Table 1), while the binding constant K_1, is barely changed. The observed leftward shift in the concentration response curve in Figure 3 is accounted for by the assumed dependence of K_o on ethanol concentration. [c] We assume that $K_o = 0.039$ (Wu et al., 1994) at all ethanol concentrations. As ethanol increases from 0 to 1.0 M, the microscopic fast desensitization rate (k_d) increases by about the same amount as the observed k_d^{max} (see Table 1) and K_1 decreases 10-fold. In this case, the decrease in K_1 is sufficient to cause the observed leftward shift in the concentration response curve in Figure 3.

Table 3: Results for a Theoretical Model Assuming Fast Desensitization Occurs Mainly from the Pre-Open State[a]

[EtOH] (M)	assumed K_o	k_{d1} (s^{-1})	k_{d2} (s^{-1})	K_1 (μM)
0	0.039	11 ± 5.4	172 ± 8.1	1250 ± 380
0.5	0.056	4.8 ± 30	1610 ± 73	520 ± 298
1.0	0.00028	0	44735 ± 1500	795 ± 110

[a] Parameters and their standard deviations are obtained from fitting the data in Figure 3 to eq 7. We assume that K_o varies with ethanol concentration in the manner obtained previously by Wu et al. (1994) and shown in the table. Due to the fact that k_{d1} is much smaller than k_{d2}, desensitization from the AR state is overshadowed by desensitization from the A_2R state, and therefore, k_{d1} cannot be determined with accuracy. k_{d2} increases dramatically with increasing ethanol concentration while K_1 remains barely unchanged.

and the model is still commonly considered (Hess, 1993). Our data provide a further test of this model, because ethanol causes such a large perturbation of the kinetics. If we assume that only closed, occupied states (AR and A_2R) contribute to desensitization ($k_{d2} \gg k_{d3}K_o$), the k_{d3} term in eq 5 can be eliminated to yield

$$-k'_d = \frac{k_{d1}2K_1[A] + k_{d2}[A]^2}{(K_1 + [A])^2 + [A]^2/K_o} \quad (7)$$

We have fit our data in Figure 3 to eq 7 assuming that K_o varies with ethanol concentration as previously determined (Wu et al., 1994) and allowing the other parameters to be determined by the data fit. The results are shown in Table 3. In the absence of ethanol, k_{d2} is greater than 10 times k_{d1}. Thus, this model predicts negligible fast desensitization from the singly occupied state. Consequently, this analysis makes no significant prediction about ethanol's action on k_{d1} because the latter's contribution to the goodness of fit is so small, but it does predict an improbably large dependence of k_{d2} on ethanol concentration of 260 M^{-1} because ethanol's open channel stabilizing action forces k_{d2} to increase in order to compensate for the decrease in A_2R concentration. Thus, if one assumes that ethanol acts on K_o, as all the available data suggest, then the action of ethanol on fast desensitization is more compatible with the model in which desensitization occurs from all states occupied by the agonist immediately after activation than one in which desensitization occurs only from pre-open states.

Mechanisms. Consideration of ethanol's actions on the open state and on fast desensitization in terms of free energy produces the insight that ethanol's underlying action on the rate constant for fast desensitization is larger than it appears to be. Thus, if ethanol were to have no other action than to stabilize the open state, the rate of desensitization from the open state would be decreased because the free energy difference between the open state and the transition state on the pathway to desensitization would be greater in the presence of ethanol. On the contrary, the rate of desensitization actually increases about 2-fold at 1 M ethanol. It follows that ethanol must have lowered the free energy of the transition state more than that of the open channel state. From Table 2A, we calculate the standard free energy change caused by ethanol in the open receptor state, A_2R^o, to be $\Delta(\Delta G^\circ_{A_2R^o}) = -10.8 \pm 0.9$ kJ mol^{-1} M^{-1}. Similarly, the standard free energy change of activation from the open state (A_2R^o) to the desensitized state (R_{fd}) caused by ethanol is $\Delta(\Delta G^\ddagger_{A_2R^o \rightarrow R_{fd}}) - \Delta(\Delta G^\circ_{A_2R^o}) = -1.43 \pm 0.02$ kJ mol^{-1} M^{-1}. Thus, ethanol changes the standard free energy of the transition state between the open state (A_2R^o) and the desensitized state (R_{fd}) by $\Delta(\Delta G^\ddagger_{A_2R^o \rightarrow R_{fd}}) = -12.2 \pm 0.9$ kJ mol^{-1} M^{-1}, a stabilizing effect that is somewhat greater than that on the open channel state.

Implications. The *Torpedo* nAcChoR is a member of the muscle subtype of nicotinic receptors, but neuronal nicotinic receptors have a high degree of homology with it also. Therefore, our work on ethanol has wider implications. We have previously estimated the general anesthetic potency of ethanol to be 330–400 mM under our temperature conditions, and inebriation would occur at one-tenth this concentration (Forman et al., 1989). Thus, at the neuromuscular junction, which operates under brief exposures to saturating agonist concentrations, inebriating ethanol concentrations would produce little effect because (i) acetylcholine opens all of the available receptors so that the ethanol-induced increase in open probability would have little additional effect, and (ii) the enhanced fast desensitization rate would be masked by the even more rapid scavenging of acetylcholine by acetylcholine esterase. However, in the central nervous system, synaptic conditions are not yet defined for nicotinic receptors. Our results allow us to predict that the highest sensitivity to ethanol would occur at a hypothetical synapse experiencing low concentrations of agonist. Here the net activity will be a complex balance between the rate of channel opening, the rate of fast desensitization, and the frequency of stimulation. The addition of ethanol to such a system would initially enhance activity because opening probability is enhanced, but subsequently this enhancement would be offset by the enhancement of the fast desensitization rate. Consistent with this, it has been reported that in stellate ganglia ethanol initially enhanced the post-synaptic response to submaximal stimulation, but when stimulation was prolonged it caused a net inhibition (Larrabee & Posternak, 1952).

In conclusion, our work identifies the open state and the transition state between the open and fast-desensitized states as two major sites of ethanol's action on the nAcChoR [this work and Wu et al. (1994)]. These actions are selective in so far as the pre-open, A_2R, and self-inhibited, A_2R^eA, states are unaffected. We cannot say whether ethanol has any other effects on the free energy surface defining the kinetics of the nAcChoR. For example, our work does not address the issue of the stability of the fast-desensitized state nor of the other transition states bordering the open state. Recently developed fast-flow patch clamp techniques (Dilger & Liu, 1992; Dilger et al., 1994) might be more appropriate for answering these questions.

APPENDIX

Determination of the Relationship between k'_d (the Slope in Figures 1 and 2) and the Microscopic Fast Desensitization Rate Constants. The equilibrium constants are defined as

$$K_1 = \frac{2[A][R_r]}{[AR]} = \frac{[A][AR]}{2[A_2R]}$$

$$K_o = \frac{[A_2R]}{[A_2R^o]} \qquad K_2 = \frac{[A][A_2R^o]}{[A_2R^eA]}$$

According to Scheme 1, the rate of formation of the agonist-induced desensitized state, R_{fd} (assumed to be a first-order process), equals the sum of the rate of desensitization of the four individual occupied states:

$$-\frac{d[R_{fd}]}{dt} = \frac{d[AR]}{dt} + \frac{d[A_2R]}{dt} + \frac{d[A_2R^o]}{dt} + \frac{d[A_2R^eA]}{dt} \quad (A1)$$

The measured decrease in flux after a period of preincubation with drugs might arise from fast desensitization and/or channel inhibition, so we rearrange eq A1 to obtain

$$-\frac{d([R_{fd}] + [A_2R^oA])}{dt} = \frac{d[AR]}{dt} + \frac{d[A_2R]}{dt} + \frac{d[A_2R^o]}{dt} = k_{d1}[AR] + k_{d2}[A_2R] + k_{d3}[A_2R^o] = [AR]\left(k_{d1} + \frac{k_{d2}[A]}{2K_1} + \frac{k_{d3}[A]}{2K_1K_1}\right) \quad (A2)$$

From the mass balance condition and using the definition of the equilibrium constants above, we obtain

$$[AR] = \frac{2[A]K_1K_o}{K_o(K_1 + [A])^2 + [A]^2}([R_T] - \{[R_{fd}] + [A_2R^oA]\}) \quad (A3)$$

Substitution of eq A3 into eq A2 gives

$$\frac{d([R_{fd}] + [A_2R^oA])}{dt} = \left(\frac{k_{d1}2[A]K_1K_o + k_{d2}[A]^2K_o + k_{d3}[A]^2}{K_o(K_1 + [A])^2 + [A]^2}\right)([R_T] - \{[R_{fd}] + [A_2R^oA]\}) \quad (A4)$$

Experimentally, we have described our data with the function $\ln(F_{Ach,t}/F_{Ctrl}) = -k'_d t + C$ (eq 3), where $F_{Ach,t}$ is proportional to $([R_T] - \{[R_{fd}] + [A_2R^oA]\})$ and F_{Ctrl} is proportional to the total receptor concentration, $[R_T]$ (see Results section). It follows that

$$\ln(F_{Ach,t}/F_{Ctrl}) = \ln(([R_T] - \{[R_{fd}] + [A_2R^oA]\})/[R_T]) = k'_d t + C \quad (A5)$$

Taking the first derivative of eq A5 yields

$$-\frac{d([R_{fd}] + [A_2R^oA])}{dt} = k'_d([R_T] - \{[R_{fd}] + [A_2R^oA]\}) \quad (A6)$$

By comparing eq A6 with eq A4, we obtain eq 5 in the Discussion section.

REFERENCES

Aoshima, H., Cash, D. J., & Hess, G. P. (1981) *Biochemistry 20*, 3467–74.

Bradley, R. J., Peper, K., & Sterz, R. (1980) *Nature 284*, 60–62.

Bradley, R. J., Sterz, R., & Peper, K. (1984) *Brain Res. 295*, 101–112.

Braswell, L. M., Miller, K. W., & Sauter, J. F. (1984) *Br. J. Pharmacol. 83*, 305–311.

Cachelin, A. B., & Colquhoun, D. (1989) *J. Physiol. (London) 415*, 159–88.

Changeux, J.-P. (1990) *Fidia Res. Found. Neurosci. Award Lect. 4*, 21–168.

Changeux, J.-P., Devillers-Thiery, A., & Chermouilli, P. (1984) *Science 335*, 1335–1345.

Colquhoun, D., & Ogden, D. C. (1988) *J. Physiol. (London) 395*, 131–159.

Dilger, J. P., & Liu, Y. (1992) *Pflugers Arch. 420*, 479–85.

Dilger, J. P., Liu, Y., Roper, J. F., & Bradley, R. J. (1994) *Biophys. J. 66*, A9.

Dreyer, F., Peper, K., & Sterz, R. (1978) *J. Physiol. 281*, 395–419.

Epstein, R., & Grundfest, H. (1970) *J. Gen. Physiol. 56*, 33–45.

Feltz, A., & Trautmann, A. (1982) *J. Physiol. 322*, 257–272.

Forman, S. A., & Miller, K. W. (1988) *Biophys. J. 54*, 149–158.

Forman, S. A., & Miller, K. W. (1989) *Biochemistry 28*, 1678–85.

Forman, S. A., Firestone, L. L., & Miller, K. W. (1987) *Biochemistry 26*, 2807–2814.

Forman, S. A., Righi, D. L., & Miller, K. W. (1989) *Biochim. Biophys. Acta 987*, 95–103.

Gage, P. W., McBurney, R. N., & Schneider, G. T. (1975) *J. Physiol. (London) 244*, 409–429.

Hess, G. P. (1993) *Biochemistry 32*, 989–1000.

Hess, G. P., Cash, D. J., & Aoshima, H. (1979) *Nature 282*, 329–31.

Huganir, R. L., Delcour, A. H., Greengard, P., & Hess, G. P. (1986) *Nature 321*, 774–776.

Katz, B., & Thesleff, S. (1957) *J. Physiol. (London) 138*, 63–80.

Krishtal, O. A., Osipchyk, Y. V., & Vrublevsky, S. V. (1988) *Neurosci. Lett. 84*, 271–276.

Larrabee, M. G., & Posternak, J. M. (1952) *J. Neurophysiol. 15*, 91.

Lee, C. Y., Chang, S. L., Kau, S. T., & Luh, S.-H. (1972) *J. Chromatogr.* **72**, 71–82.

Linder, T. M., Pennefather, P., & Quastel, D. M. (1984) *J. Gen. Physiol. 83*, 435–468.

Miller, K. W., Firestone, L. L., & Forman, S. A. (1987) *Ann. N.Y. Acad. Sci. 492*, 71–87.

Miller, K. W., Wood, S. C., Forman, S. A., Bugge, B., Hill, W. A. G., & Abadji, V. (1991) *Ann. N.Y. Acad. Sci. 625*, 600–615.

Nelson, D. J., & Sachs, F. (1981) *Biophys. J. 33*, 121a.

Ochoa, E. L. M., Chattopadhyay, A., & McNamee, M. G. (1989) *Cell. Mol. Biol.* 9, 141–178.

Sakmann, B., Patlak, J., & Neher, E. (1980) *Nature 286*, 71–73.

Sine, S. M., Claudio, T., & Sigworth, F. J. (1990) *J. Gen. Physiol. 96*, 395–437.

Trussell, L. O., & Fischbach, G. D. (1989) *Neuron 3*, 209–218.

Udgaonkar, J. B., & Hess, G. P. (1986) *J. Membr. Biol. 93*, 93–109.

Walker, J. W., Takeyasu, K., & McNamee, M. G. (1982) *Biochemistry 21*, 5384–9.

Wu, G., Tonner, P. H., & Miller, K. W. (1994) *Mol. Pharmacol. 45*, 102–108.