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Ethanol increases agonist affinity for nicotinic receptors from *Torpedo*

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The presence of ethanol increases the apparent affinity with which acetylcholine and carbamylcholine elicit $^{86}Rb^+$ flux from *Torpedo* nicotinic acetylcholine receptor-rich vesicles at 4°C. Affinity increased exponentially with ethanol concentration, reaching nearly 200-fold by 3.0 M ethanol without sign of saturation. At submaximal agonist concentrations 50–100 mM ethanol enhanced flux by 15–35%, but the maximum agonist-induced flux was unaffected in quenched-flow assays. The effect was independent of the agonist and of the time over which flux was measured (5 ms to 10 s), indicating that ethanol acts before agonist-induced desensitization occurs. Ethanol also caused an increase in the apparent affinity with which acetylcholine caused fast desensitization. This affinity increase was equal to that for flux-response curves, but the maximum fast desensitization rate was increased 50% at 0.5 M ethanol. This was the most pronounced of ethanol's actions and has not been reported before. Prolonged preincubation with 1.0 M ethanol alone reduced agonist-induced flux activity by only 25%. The rate of agonist-induced slow desensitization was also increased, but neither of these effects was as marked as those on fast desensitization and cation flux.

Introduction

Short-chain alcohols, including ethanol, have been shown to enhance nAcChoR-mediated cation flux at the neuromuscular junction in a number of electrophysiological studies. Ethanol slows the rate of miniature endplate current (mepc) decay [1,2], and decreases the frequency of the endplate noise power spectra [1,3], indicating that the rate of cation channel closing is slowed. In isolated skeletal muscle, ethanol increases skeletal muscle twitch tension (both directly and indirectly stimulated) [4,5], yet preliminary single-channel studies indicate that nAcChoR channel conductance is not altered by ethanol [6]. In contrast, long-chain alcohols and a wide variety of other anesthetic agents inhibit neuromuscular junction depolarization by increasing mepc decay rates [7]. Thus, the short-chain alcohols appear to have a unique, specific effect on the nAcChoR receptor-channel complex.

Several lines of evidence suggest that ethanol may act by modulating the affinity of cholinergic agonists for channel activation sites on the nAcChoR. Linder et al. [8] proposed that the slower mepc decay rates observed with ethanol reflect a decreased rate of dissociation of acetylcholine from its receptor. Using electrophoretic techniques for rapid agonist application [9] on voltage-clamped endplates, Bradley et al. [3,10] showed that ethanol decreased the apparent acetylcholine dissociation constant. In a preliminary report [11], we presented evidence that ethanol increases the apparent affinity of carbamylcholine for *Torpedo* nAcChoR, a receptor which is highly homologous with the vertebrate neuromuscular junction receptor.

In addition, ethanol has been shown to inactivate nAcChoR in the absence of agonist. Ethanol stabilizes an inactive nAcChoR state, characterized by an increase in agonist affinity, as has been demonstrated using various ligands as probes of the receptor's state [11–13]. However, effects of ethanol on *agonist-induced* desensitization on either the minute time scale (slow desensitization) or the subsecond time scale (fast desensitization) have not been reported.

In this paper, we present studies of the effects of ethanol on the nAcChoR cation channel from *Torpedo*. Several agonist-triggered receptor functions were studied

Abbreviations: nAcChoR, nicotinic acetylcholine receptor; α-BTX, α-bungarotoxin; GABA, γ-aminobutyric acid, mepc, miniature endplate current; NMDA, *N*-methyl-D-aspartate.

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using $^{86}Rb^+$ flux measurements in sealed *Torpedo* electroplaque vesicles, including activation of cation channels, slow desensitization, and fast desensitization. Because quenched-flow techniques can produce rapid, precise drug concentration jumps in suspensions of *Torpedo* vesicles they have a clear advantage over electrophysiological techniques for studying agonist concentration-response curves [14] and fast desensitization.

Materials and Methods

Preparation of Torpedo postsynaptic membranes. Postsynaptic membranes from freshly dissected electroplaques of *Torpedo nobiliana* (Biofish Associates, Georgetown, MA) were prepared at 4°C, essentially as described by Krodel et al. [15]. Aliquots of electroplaque tissue were homogenized with an equal weight of aqueous 0.02% NaN_3 solution containing 0.1 mM phenylmethylsulfonyl fluoride to inhibit proteolysis. The homogenate was centrifuged at $5400 \times g$ for 10 min to pellet large tissue fragments and the supernatant was centrifuged at $15000 \times g$ for 90 min to pellet membrane fragments. Membranes were further purified by sucrose density gradient centrifugation. Fractions rich in nAcChoR were pooled and resuspended in a small volume of *Torpedo* physiological saline (250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM NaP_i, and 0.02% NaN_3, pH 7.0). The suspensions contained 5 to 10 mg protein/ml (assayed by the method of Lowry et al. [16], using bovine serine albumin as standard) and 7 to 15 μM in [^{3}H]acetylcholine binding sites (assayed as described previously [17]) and were kept frozen in liquid N_2 for up to 6 months and thawed within 48 h of use.

Measurement of nAcChoR cation channel function. Cation channel function was determined by measuring agonist-induced $^{86}Rb^+$ efflux or influx at 4°C in sealed *Torpedo* electroplaque vesicles which, for efflux studies, were loaded with $^{86}Rb^+$ by overnight incubation [17]. The number of active receptor-channel complexes was reduced by preincubation with sufficient α-bungarotoxin (α-BTX) to prevent full equilibration of $^{86}Rb^+$ across vesicle membranes during flux assays. Efflux or influx of $^{86}Rb^+$ was assayed by two techniques. For flux times of 10 s or longer, solutions were mixed by hand and flux stopped by filtration. For shorter periods, a quenched-flow technique was used and flux was stopped with procaine [17] and the intravesicular $^{86}Rb^+$ separated by passage over a cation exchange resin as described previously [18]. Ethanol was added to vesicles at the same time as agonist so that the time that vesicles were exposed to ethanol was minimal. Preincubation of vesicles with up to 1.5 M ethanol for 10 s prior to addition of agonists had no effect on flux assays. Channel activity was stimulated with either acetylcholine or carbamylcholine, an acetylcholine analogue that is not hydrolyzed by acetylcholinesterase. When acetylcholine was used, $^{86}Rb^+$-loaded vesicles were treated with 0.1 mM diisopropylfluorophosphate for 20 min prior to flux assays to inhibit acetylcholinesterase. Gross agonist-stimulated $^{86}Rb^+$ efflux counts (CPM(Ag,t)) were corrected for passive, time-dependent $^{86}Rb^+$ 'leakage' from sealed vesicles (CPM(leak,t)). The corrected efflux response is expressed as F_A, the percentage of non-leak $^{86}Rb^+$ counts released [17]

$$F_A = \frac{\text{CPM(Ag},t) - \text{CPM(leak},t)}{\text{CPM(total)} - \text{CPM(leak},t)} \times 100\% \quad (1)$$

Analysis of ethanol-enhanced $^{86}Rb^+$ leak from sealed vesicles. Any ethanol-induced enhancement of $^{86}Rb^+$ leak from sealed vesicles during a 10-s exposure was detected as an increase in filtrate radioactivity above the leak level without ethanol present and analyzed similarly to agonist-induced efflux (Eqn. 1):

$$\text{leak enhancement} = \frac{\text{CPM(Alc},t) - \text{CPM(0 Alc},t)}{\text{CPM(total)} - \text{CPM(0 Alc},t)} \times 100\% \quad (2)$$

Measurement of slow agonist-induced desensitization kinetics. Vesicles (α-BTX treated, 0.5 ml) were mixed manually with an equal volume of *Torpedo* physiological solution containing carbamylcholine, ethanol, or both, and the mixture was incubated for up to 30 min at 4°C. At various times during the incubation, a 50 μl aliquot was removed and mixed with an equal volume of 10 mM carbamylcholine solution containing $^{86}Rb^+$ at 50 μCi/ml. After 10 s, $^{86}Rb^+$ influx was halted by mixing with 100 μl of 100 mM procaine. 175 μl of the final vesicle suspension was applied to a small (1 ml) cation exchange column and vesicles were eluted with 220 mM sucrose (for details, see Forman and Miller, [18]). CPM(leak) was measured in an identical fashion, but without agonist present. CPM(total) was established by overnight incubation of vesicles with $^{86}Rb^+$ solution before the addition of procaine and passage over cation exchange resin. Flux activity after preincubation for a time, was expressed as the fraction of that present without any preincubation step ($F_A(0)$). Plots of fractional flux activity vs. time were analyzed by fitting data to a single-exponential function using non-linear least squares:

$$\frac{F_A(t)}{F_A(0)} = \left(1 - \frac{F_A(\infty)}{F_A(0)}\right) \times \exp(-k_s \times t) + \frac{F_A(\infty)}{F_A(0)} \quad (3)$$

where $F_A(\infty)/F_A(0)$ is the fraction of activity remaining at equilibrium and k_s is the rate of slow desensitization.

Measurement of fast agonist-induced desensitization kinetics. Fast desensitization rates were determined using a method analogous to that described for slow desensitization, but an automated pulsed quenched-flow device was used to control preincubation and $^{86}Rb^+$ influx

periods on a millisecond time scale (described in Ref. 18).

Reversibility of ethanol actions. The reversibility of the effects of a 10-s exposure to 1.0 M ethanol in the absence of agonists was tested using the filtration technique to measure $^{86}Rb^+$ efflux. To 100 μl of $^{86}Rb^+$-loaded vesicles were added 200 μl of *Torpedo* physiological saline containing 1.5 M ethanol (final concentration 1.0 M). After incubating for 10 s, vesicles were diluted 10-fold in *Torpedo* physiological saline (i.e., back-diluted to 0.1 M ethanol) and then 1 ml aliquots were mixed with either 10 μl of carbamylcholine to stimulate flux (final carbamylcholine concentration = 56 μM) or 10 μl *Torpedo* physiological saline (to measure leak) for another 10 s before filtration. One set of control experiments followed the same protocol, except vesicles were diluted 10-fold in *Torpedo* physiological saline with 1.0 M ethanol (final concentration 1.0 M) prior to leak and flux determinations. Another set of control experiments were performed by the same method, but with 0.1 M ethanol throughout.

Preparation of ethanol solutions. Solutions of ethanol in *Torpedo* physiological saline were prepared by weighing ethanol into *Torpedo* physiological saline in partially filled volumetric flasks and adjusting with *Torpedo* physiological saline to final volume. At room temperature, ethanol concentrations over 2.0 M caused *Torpedo* physiological saline solutions to become cloudy (probably due to calcium phosphate precipitation). This was prevented by using ice-cold *Torpedo* physiological saline and solutions prepared in this fashion were used immediately in the cold room (4°C). Ethanol and agonist solutions were mixed just before adding vesicles in order to reduce evaporative losses. Gas chromatography determined the losses to be less than 3% at 4°C.

Chemicals. Diisopropylfluorophosphate was from Aldrich Chemical Co. (Milwaukee, WI), α-bungarotoxin (α-BTX), buffer reagents, acetylcholine chloride, carbamylcholine chloride, and procaine hydrochloride were from Sigma Chemical Co. (St. Louis, MO). [^{3}H]Acetylcholine and ^{86}RbCl were from New England Nuclear (Boston, MA). Anhydrous ethanol (≥ 99.9% purity) was from Pharmco (Dayton, NJ).

Results

Ethanol effects on passive $^{86}Rb^+$ leak

Since ethanol perturbs membranes [19], its effect on passive $^{86}Rb^+$ 'leakage' from *Torpedo* vesicles during a 10-s exposure was tested first. Significant increases in leakage after 10 s (more than 5% leak enhancement) were only observed at or above 1.5 M ethanol. Thus, subsequent flux studies were only corrected for enhanced $^{86}Rb^+$ leak when the ethanol concentration was 1.5 M or greater. Above 1.5 M ethanol, leak counts after 10 s increased rapidly and each F_A assay required correction with a matched ethanol-enhanced $^{86}Rb^+$ leak measurement. Above 3.0 M ethanol the leak after 10 s was about 25% of the total intravesicular $^{86}Rb^+$ counts, and the variation in both leak and agonist-induced efflux measurements increased. Under these conditions, the sensitivity of flux assays to changes in channel activity was deemed unacceptable for quantitative analysis of concentration-response data. Therefore, the maximum ethanol concentration used was 3.0 M.

Ethanol effects on agonist concentration-response curves

The effects of ethanol were determined both on carbamylcholine stimulated 10-s flux and on acetylcholine stimulated flux using quenched-flow assays. Leak-corrected flux responses were analyzed by fitting to a logistic equation:

$$F_A = F_A(\max) \times \left(\frac{A^{N_1}}{A^{N_1} + K_A^{N_1}} \right) \quad (4)$$

where A is the agonist concentration, F_A(max) is the the maximum agonist-stimulated $^{86}Rb^+$ flux (usually at 5 mM carbamylcholine or 1 mM acetylcholine), K_A is the 50% activating concentration, and N_1 is the Hill coefficient.

It was more convenient to study the effects of ethanol on apparent K_A over a wide range of concentrations using the 10 s filtration assays. These involved a shorter overall exposure to ethanol than did quenched-flow experiments because the time between quenching and passing the vesicles over the cation exchange resin exceeded 10 s. Thus the 10-s assay minimized the leak correction enabling concentrations of ethanol up to 3.0 M to be studied. Table I summarizes the parameters derived by fitting the data to Eqn. 4 by non-linear least-squares methods [11]. The apparent K_A values in Table I decrease continuously with increasing ethanol concentration without ever achieving a plateau. At 3.0 M ethanol a 180-fold decrease in apparent K_A was observed. The Hill coefficients remained unchanged up

TABLE I

Fitted parameters from 10 s carbamylcholine concentration-response curves

Parameters were derived by non-linear least-squares fitting of leak-corrected flux data (at least 12 points in each curve) to Eqn. 4. A plot of some of the data has been given previously by Miller et al. [11].

[Ethanol] (M)	F_A(max) (%)	K_A (μM)	N_1
0.0	35.3 ± 0.3	120 ± 3	1.5 ± 0.1
0.3	31.1 ± 0.3	52 ± 2	1.6 ± 0.1
0.6	30.2 ± 0.3	24 ± 1	1.3 ± 0.1
0.9	30.5 ± 0.4	13.3 ± 0.8	1.3 ± 0.1
1.5	31.1 ± 0.4	4.3 ± 0.3	1.1 ± 0.1
2.7	31.3 ± 0.5	0.8 ± 0.1	0.7 ± 0.1
3.0	31.9 ± 0.8	0.67 ± 0.08	0.7 ± 0.1

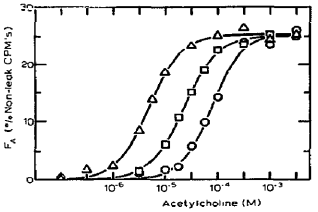


Fig. 1. Effects of ethanol on acetylcholine concentration-response curves measured using rapid quenched-flow. The effects of ethanol on acetylcholine-induced $^{86}Rb^+$ efflux were studied in α-BTX treated vesicles. Data were fitted to Eqn. 4 by non-linear least squares (see Results). Concentration-response curves with flux integrated over 30 ms (0.5 M ethanol) and over 5 ms (1.0 M ethanol). The K_A of the control was unaffected by the integration time and F_A(max) was unaltered by ethanol ($p \approx 0.5$). For convenience the two sets of curves have been normalized to have the same experimental F_A(max) in the diagram. Fitted parameters for the lines shown are as follows:

[Ethanol]	K_A (μM)	N_1
None (○)	79 ±7	1.5±0.2
0.5 M (□)	23.3±0.8	1.3±0.1
1.0 M (△)	5.0±0.3	1.4±0.1

to approx. 1 M, but above this a decrease was observed. Table I also shows that the F_A(max) was decreased by 15% over the whole concentration range studied (0.3–3.0 M). This small effect on F_A(max), which is caused by enhanced fast desensitization (see below and Discussion), had little influence on the overall analysis.

The leftward shift of agonist concentration-response curves was examined further in quenched-flow experiments, which showed that the maximum flux elicited by either acetylcholine or carbamylcholine was unaffected by ethanol in the range 5–45 ms, showing that the small decrease seen in the 10-s experiments develops over an intermediate time scale. Fig. 1 shows that the decrease in acetylcholine's apparent dissociation constant, which occurs without change in Hill coefficient, was 3.4-fold at 0.5 M ethanol (30 ms integration) and 16-fold at 1.0 M (5 ms integration).

For comparison, we define a parameter, SC_{50}, as the concentration at which $K_A^{alc}/K_A^0 = 0.5$. Based on quenched-flow acetylcholine response curves, the SC_{50} for ethanol is 250 ± 30 mM and for the 10-s assay with carbamylcholine it is 290 ± 20 mM. Thus, the increase in affinity is independent of both the agonist and the time scale of the experiment.

Effect of low ethanol concentrations

Although the extent of the increase of affinity observed in the above studies is quite large, the ethanol concentrations used are well above the range observed in human subjects (up to 100 mM; [20]). However, ethanol's potency decreases with decreasing temperature. For example, in the tadpole the anesthetic concentration increases from 200 mM at 20°C to 340 mM at 10°C and in the frog from 140 at 30°C to 330 mM at 3°C [21]. In the nAcChoR both 10-s and quenched-flow assays indicate that when the flux is stimulated by submaximal concentrations of agonists (Table II), it is enhanced at sub-anesthetic concentrations as low as 50 mM ethanol ($p \leq 0.02$).

TABLE II

Enhancement of carbamylcholine-induced $^{86}Rb^+$ flux by ethanol at 4°C

Values shown are the ratios of averaged flux measurements ($n = 4$) measured in the presence of the indicated ethanol concentration to control measurements made without ethanol.

[Ethanol]	10 s	20 ms
	$F^{alc}_{10\mu M}/F^0_{10\mu M}$	$F^{alc}_{220\mu M}/F^0_{220\mu M}$
50	1.20±0.05	1.14±0.07
100	1.48±0.05	1.45±0.05
200	2.20±0.10	2.28±0.11

Ethanol effects on slow desensitization

In the prolonged presence of low agonist concentrations, even those below that at which cation-flux activity is observed, nAcChoR undergoes slow desensitization. Fig. 2 shows that ethanol increases both the rate

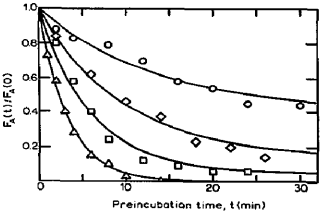


Fig. 2. Effect of ethanol on carbamylcholine-induced slow desensitization. *Torpedo* vesicles (≈ 5% nM in total [^{3}H]acetylcholine sites and blocked with α-BTX to inactivate 80% of sites) were preincubated with 50 nM carbamylcholine and varying amounts of ethanol before measuring $^{86}Rb^+$ influx for 10 s in the presence of 5 mM carbamylcholine. Residual flux activity ($F_1(t)$) is shown as the fraction of flux activity measured without preincubation ($F_A(0)$). Data were fitted to Eqn. 3 by non-linear least squares with $F_A(\infty)$ fixed at the value measured after more than 30 min of incubation. Fitted parameters for the lines shown are as follows:

[Ethanol]	$F_A(\infty)/F_A(0)$	k_s (min^{-1})
None (○)	0.40±0.03	0.073±0.013
0.25 M (◇)	0.14±0.03	0.105±0.016
0.5 M (□)	0.05±0.02	0.171±0.023
1.0 M (△)	0.00±0.02	0.304±0.015

and extent of slow desensitization over a period of minutes. Whereas preincubation with 50 nM carbamylcholine alone inactivates about 60% of receptors with a k_s of 0.073 min^{-1}, a combination of 50 nM carbamylcholine plus 1 M ethanol fully inactivates receptors 4-fold faster with a k_s of 0.304 min^{-1}.

Because of the long exposures to ethanol in slow desensitization experiments, we performed control studies to measure the effects of long preincubations with ethanol alone. Previous studies, using [^{3}H]acetylcholine binding assays indicate that 0.7 M ethanol alone causes 50% inactivation (conversion to high affinity state) of nAcChoR after a 30 min incubation at 4°C [11]. Using $^{86}Rb^+$ flux to directly assess nAcChoR activity, we found that 0.5 M ethanol caused no inactivation at up to 30 min, while 1.0 M ethanol caused a slow drop in flux activity to 75 ± 5% of initial after 30 min. Thus, ethanol's main effect is to enhance agonist-induced desensitization, but a small part of the inactivation observed with 50 nM carbamylcholine plus 1 0 M ethanol may be attributed to the action of ethanol alone.

Ethanol effects on fast desensitization

In addition to increasing the rate of slow desensitization, k_s, we established using pulsed quenched-flow that 0.5 M ethanol enhances fast desensitization which occurs on a subsecond time scale. Fig. 3A shows semilog plots of fast desensitization induced by increasing concentrations of acetylcholine. At all acetylcholine concentrations studied, equilibrium desensitization (preincubation for 10 min) eliminated all flux activity. The fast desensitization rates (k_d values) derived from analysis of data in Fig. 3A are plotted against acetylcholine concentration in Fig. 3B and are fitted to a logistic equation analogous to Eqn. 4:

$$k_d = k_d(\max) \times \left(\frac{A^{N_D}}{A^{N_D} + K_D^{N_D}} \right) \quad (5)$$

where K_D is the acetylcholine concentration causing desensitization at a rate of $k_d(\max)/2$ and N_D is the Hill coefficient.The dashed line in Fig. 3B represents a fit to Eqn. 5 for desensitization rates measured in the absence of ethanol. The K_D in the presence of 0.5 M ethanol is 3.3-fold lower than that in its absence, and $k_d(\max)$ is 50% higher in the presence of ethanol.

Reversibility of ethanol effects

In order to determine whether 10-s exposures to 1 M ethanol caused irreversible functional effects of nAcChoR, reversibility experiments were performed. 1 M ethanol increased the flux stimulated by 56 μM carbamylcholine in 10 s ($F_{56\mu M}$) from 15 ± 3% to 45 ± 3%. Ten-fold back dilution (to 0.1 M ethanol) into a carbamylcholine containing solution after a 10-s ethanol exposure in the absence of carbamylcholine resulted in $F_{56\mu M} = 22 \pm 2\%$, while a control experiment where vesicles were exposed to 0.1 M ethanol for both a 10 s preincubation and the carbamylcholine test stimulation gave $F_{56\mu M} = 23 \pm 2\%$. This result indicates complete reversibility of the flux enhancement caused by a 10 s exposure to 1.0 M ethanol.

Discussion

Advantages of flux studies

The data presented above extend previous electrophysiological studies of ethanol actions at the neuro-

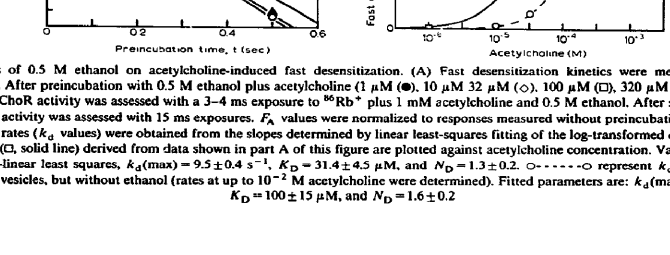


Fig. 3. Effects of 0.5 M ethanol on acetylcholine-induced fast desensitization. (A) Fast desensitization kinetics were measured by pulsed quenched-flow. After preincubation with 0.5 M ethanol plus acetylcholine (1 μM (●), 10 μM 32 μM (◇), 100 μM (□), 320 μM (○), or 1 mM the remaining nAcChoR activity was assessed with a 3–4 ms exposure to $^{86}Rb^+$ plus 1 mM acetylcholine and 0.5 M ethanol. After some of the longer preincubations activity was assessed with 15 ms exposures. F_A values were normalized to responses measured without preincubation ($F^{0,0}_{1mM;T}$). Fast desensitization rates (k_d values) were obtained from the slopes determined by linear least-squares fitting of the log-transformed data as shown. (B) The k_d values (□, solid line) derived from data shown in part A of this figure are plotted against acetylcholine concentration. Values were fitted to Eqn. 5 by non-linear least squares, $k_d(\max) = 9.5 \pm 0.4\ s^{-1}$, $K_D = 31.4 \pm 4.5$ μM, and $N_D = 1.3 \pm 0.2$. ○- - - - -○ represent k_d values measured using the same vesicles, but without ethanol (rates at up to 10^{-2} M acetylcholine were determined). Fitted parameters are: $k_d(\max) = 6.5 \pm 0.3\ s^{-1}$, $K_D = 100 \pm 15$ μM, and $N_D = 1.6 \pm 0.2$

muscular junction in two significant directions. Firstly, the ability to change the effective agonist concentration in less than a millisecond and to measure cation flux integrated over a few milliseconds means that, in contrast to physiological studies, concentration-response curves can be obtained before the development of desensitization in *Torpedo* vesicle suspensions. Secondly, our methods enable exposure to ethanol to be very brief, enabling concentrations as high as 3 M to be studied. In comparison, physiological studies required ethanol exposure times of 20 min or more, causing irreversible loss of agonist-induced currents at ethanol concentrations over 1 M [10]. Although Boyd and Cohen [22] observed irreversible loss of [^{3}H]acetylcholine binding to nAcChoR from *Torpedo* with long exposures to propanol and butanol, with the brief exposures possible in our work the maximum agonist-induced flux did not change between 0.3 and 3.0 M ethanol, nor did the passive leakage of $^{86}Rb^+$ through vesicle membranes increase excessively.

Ethanol enhances apparent agonist affinity for flux and fast desensitization

A remarkable degree of enhanced cation flux activity is seen at low, but not high, agonist concentrations when ethanol is added to $^{86}Rb^+$ flux assays in *Torpedo* nAcChoR-rich vesicles, confirming and extending previous observations from electrophysiological studies as well as our own preliminary data [11].

This ethanol-induced enhancement of flux at low agonist concentrations occurs because of an increase in the apparent affinity of the agonists for opening channels, a conclusion similar to that obtained from acetylcholine dose-response curves determined by quantitative electrophoretic studies on the vertebrate neuromuscular junction [10]. In the latter study, 400 mM ethanol at 18°C reduced the apparent acetylcholine dissociation constant (K_A) by half, while higher concentrations caused irreversible loss of function. Our 10 s and quenched-flow $^{86}Rb^+$ flux studies at 4°C give similar reductions of K_A at 290 and 250 mM, respectively. Considering the well known problems of obtaining K_A values from physiological experiments and the temperature difference, there is satisfactory agreement between our work and that of Bradley et al. [10].

The flux studies demonstrated the increase in apparent agonist affinity ($1/K_A$) over a wide range of ethanol concentrations. This leftward shift in the concentration-response curves was analysed as the ratio of K_A in the presence of ethanol (K_A^{alc}) to that in its absence (K_A^0) for both the 10 s and quenched-flux assays. Fig. 4 shows that (K_A^{alc}/K_A^0) decreases exponentially as the ethanol concentration increases up to 1.0 M, where an approximately 10-fold reduction is observed, independent of which technique was employed. At higher ethanol concentrations, the plot deviates

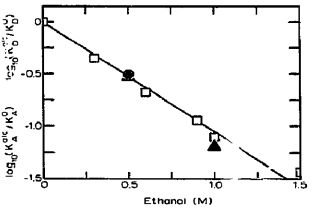


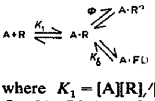
Fig. 4. Ethanol dependence of apparent agonist affinities. Fitted K_A values (K_A^{alc} values) from ethanol-shifted carbamylcholine concentration-response curves for 10-s exposures (□; Table I) and acetylcholine concentration-response curves for brief exposures (▲; Fig. 1) were normalized to control K_A values in the absence of alcohol (K_A^0 values) and plotted on a log scale against ethanol concentration. In addition, the ratio of the fitted parameter, K_D, from fast desensitization rate analysis (Fig. 3) in the presence of 0.5 M ethanol (K_D^{alc}) to that without ethanol (K_D^0) is plotted on the same scale (○). All the data were fitted to a straight line by linear least squares, resulting in a slope of -1.10 ± 0.08 M^{-1}. This line predicts that $SC_{50} = 275 \pm 30$ mM.

slightly from linearity, but there is no identifiable saturation in the leftward-shift up to 3.0 M ethanol (Table I; and Ref. 11). Thus, our data provide no evidence that the effect of ethanol on K_A saturates.

Ethanol also causes a leftward shift in the concentration-response curve for fast desensitization (Fig. 3B) for which the ratio K_D^{alc}/K_D^0 (Eqn. 5) at 500 mM ethanol has an almost identical value to (K_A^{alc}/K_A^0) for 30 ms integrated flux (Fig. 4).

Kinetic interpretation

In order to appreciate the significance of the ethanol-induced decrease in the apparent K_A as well as the effect of desensitization on our results, it is helpful to consider a simplified scheme depicting the mechanisms of nAcChoR channel activation and fast desensitization:



where $K_1 = [A][R]/[A \cdot R]$, $K_\delta = [A \cdot R]/[A \cdot FD]$ and $\Phi = [A \cdot R]/[A \cdot R^*]$.

Agonist binding to the resting state of the receptor, R, is thought to lead rapidly to a pre-open state, A · R, in rapid equilibrium with the open-channel state, A · R*, and more slowly (about a second) with the fast-desensitized state, A · FD [23,24]. This simplified scheme is sufficient to examine the changes induced by ethanol, but it ignores the fact that occupation by two molecules of acetylcholine are required to open the channel and that fast desensitization may proceed from open as well

as pre-open receptors [18]. Slow desensitization is also ignored here because the time it takes to develop is long compared to either flux assay.

According to this scheme, the agonist-induced flux concentration-response curve is given by the state function for $A \cdot R^o$. This includes contributions from K_1, K_δ and Φ. In addition, as is well known, fast desensitization will influence the 10-s flux assay: in fact, it causes an apparent shift of the agonist-response curve to lower concentrations [25].

The ethanol-induced leftward shift of the concentration-response curves for agonist-induced cation flux integrated over 10 s could be attributed a priori to a number of possible mechanisms (see Eqn. 6), including: (a) increasing agonist affinity, $1/K_1$; (b) displacement of the pre-open to open state equilibrium towards the open state (equivalent to decreasing Φ); (c) decreasing fast desensitization rates which would increase K_δ, and (d) increasing single-channel conductance.

Of these putative mechanisms, the latter, d, is ruled out by single-channel studies which show that ethanol does not change channel conductance [6], while effects on fast desensitization, c, are unlikely since the leftward shift can be observed at 5 ms while the maximum desensitization rate we observed was less than 10 s^{-1}. However, distinguishing between the remaining two explanations is more difficult.

At first sight, explanation b also seems unlikely because decreasing Φ would lead to an increase in the maximum observed flux. However, single-channel studies show that the ratio $(1/\Phi)$ of microscopic channel opening rates to closing rates is high (at least 32 for acetylcholine [24]). Thus, at saturating agonist concentrations, the probability of non-desensitized channels being open is ≥ 0.97 and displacing the equilibrium further towards the open state would result in little additional observable flux. Therefore, explanation b cannot be ruled out on the basis of flux studies. Unfortunately, single-channel studies are complicated by time dependent effects on kinetics, and the preliminary data that have been published to date do not settle this question.

The conclusion that a decrease in K_1 explains the leftward shift in the agonist concentration-flux curves is reinforced by our observation that ethanol causes the same degree of leftward shift for fast desensitization as it does for flux (Fig. 4). Closer inspection shows that the desensitization rate curves with and without 0.5 M ethanol (Fig. 3) parallel their respective acetylcholine flux response curves (Fig. 1) closely: K_D values are close to K_A values, while the Hill coefficients for the two sets of curves are identical. Considering the symmetry in Eqn. 6, the simplest explanation is that both shifts result from a common perturbation at the binding step.

Thus, our concentration-response data tend to favor an ethanol-induced increase in apparent agonist affinity, but they do not rule out an additional effect on the closing rate. An effect on K_1 might reflect an increase in the on-rate for acetylcholine binding or a decrease in the off-rate for acetylcholine dissociation. The on-rate is considered to be diffusion limited, but it might be further enhanced if the relatively long range electrostatic forces between the receptor and the agonist were strengthened in the presence of ethanol. Although ethanol at the concentrations employed here would indeed reduce the dielectric constant of the aqueous medium, this effect would be too small to explain our data. Thus, a decrease in off-rate is most probable [8].

Ethanol increases fast desensitization rates

Although the decrease in K_1 may explain the equal leftward shift in the fast desensitization and flux concentration-response curves (Fig. 4), it does not explain why the maximum apparent rate of fast desensitization increases while the maximum flux does not. In this respect, ethanol appears to act unsymmetrically on Eqn. 6. The observed fast desensitization rate, k_d, is a function of the concentration of the pre-open state, AR, and of the rate constants for the $AR \rightleftharpoons A \cdot FD$ transition. At all saturating agonist concentrations, the concentration of $A \cdot R$ will remain unchanged on addition of ethanol, suggesting that K_δ is decreased through an action on the forward or backward rate constant or both. More detailed studies would be required to resolve whether ethanol acts separately on K_1 and K_δ, or whether, unlike Φ, the initial value of K_δ is such that the maximum k_d will be observed to increase when the leftward shift occurs. The latter possibility seems less likely because it suggests that ethanol acts equally on Φ and K_δ.

The increase in the maximum value of k_d does explain why ethanol caused a 15% drop in $F_A(\max)$ in 10-s concentration-response curves (Table I), while no change was seen in millisecond flux assays (Fig. 1). Thus, if one assumes an initial maximum $^{86}Rb^+$ efflux rate (k_f^0) of 15 s^{-1} (estimated as described in Forman and Miller [18]) at 1 mM acetylcholine in both the presence and absence of ethanol, Eqn. 7 [25] can be used to calculate integrated efflux in the presence of an exponential fast desensitization occurring at a rate of k_d:

$$F_A(t) = F_A(\max) \times \left(1 - \exp\left(\frac{-k_f^0[t - \exp(-k_d t)]}{k_d}\right)\right) \quad (7)$$

This equation predicts that after 10 s, $F_A(\max)$ will drop 13% when k_d is increased from 6.5 s^{-1} (no ethanol) to 9.5 s^{-1} (0.5 M ethanol). Within experimental error, this is the same as the observed drop in 10-s flux when ethanol was added to 1 mM acetylcholine.

Ethanol enhances slow desensitization

Our data show that, when flux activity is used to assay remaining activity, preincubation with 1 M ethanol alone causes little desensitization over 30 min. This contrasts with previous studies which used various ligands, such as ^{125}I-α-bungarotoxin [12], [^{3}H]acetylcholine [11] and a channel blocker, [^{3}H]histrionicotoxin [13], to probe the receptor's state. For example, preincubation for 30 min with 700 mM ethanol converted half the resting-state receptors to the desensitized state, as estimated by a subsequent 5-s test exposure to concentrations of [^{3}H]acetylcholine low enough to bind only to high affinity receptors [11]. Such discrepancies may occur because ethanol's unusual effect on agonist binding affinity may lead to an overestimation of desensitization when standard ligand binding assays are used [11,12], a possibility that merits further attention.

Whereas 0.5 and 1.0 M ethanol alone caused 0 and 25% desensitization in 30 min, in the presence of low agonist concentration the desensitization was 95 and 100%, respectively. This suggests that ethanol interacts with the agonist occupied desensitized state more strongly than the pre-existing desensitized state. Consistent with this explanation, the apparent slow desensitization rate (k_s, Fig. 2) increased linearly with ethanol concentration in the range 0–1.0 M.

Molecular mechanisms of ethanol's action

Our data enable us to comment on some molecular mechanisms that have been proposed to account for the action of ethanol at acetylcholine receptors.

Gage et al. [1] suggested that short-chain alkanols, including ethanol, reduce the membrane dielectric constant near nAcChoR, which slows the rate-limiting reorientation of a protein dipole within the transmembrane electric field during channel closure. Arguing that the change in membrane dielectric constant was a linear function of the weight fraction of membrane-phase alcohol, this model accounted for the exponential relationship between mepc lifetime and ethanol concentration. The hypothesis was indirectly supported by evidence that ethanol inhibits a crustacean neuromuscular junction with a voltage sensitivity opposite to that observed for vertebrates, which the model attributes to reversal of the gating dipole orientation [26].

It is interesting to note that when the data of Gage et al. [1] is replotted as $\log_{10}$(mepc decay time constant) vs. the ethanol concentration, a slope of 1.5 M^{-1} is obtained. If we calculate $\log_{10}(F_A)$ for a low carbamylcholine concentration (2 μM) from 10-s agonist-response curves using the parameters in Table I and plot the results against ethanol concentration up to 2.7 M, the result is a straight line with slope = 1.52 M^{-1} (r = 0.97). Thus, the two sets of data seem to be closely related, yet our work provides little indication that the channel closing rate is altered by ethanol. Indeed it is possible that Gage's conclusion that ethanol affects the channel closing rate may be incorrect, since a decrease of either the channel closing rate or the agonist dissociation rate will lead to a slower observed rate of mepc decay [8,27,28]. The specific issue of whether ethanol microscopically alters the agonist off-rate, resulting in longer channel opening bursts, or the intra-burst distribution of open and closed times (or both) could be profitably studied using single-channel methods. In any case, the physical basis of the Gage mechanism requires that a transmembrane electric field exist for the action of ethanol to occur, yet in our work both flux and fast desensitization are enhanced by ethanol in the absence of an applied transmembrane voltage.

Bradley et al. [10] proposed a 'hydrophobic patch' site within the AcChoR ion channel, similar to that at which alkanols are thought to inhibit firefly luciferase [29]. In the Bradley model, ethanol binds to the open-channel state without inhibiting cation flux, thus increasing the apparent agonist affinity by 'uncompetitive' stabilization of the open-channel state (i.e., increased channel lifetime, as in the Gage model). Bradley et al. [10] extended their model by assuming that long- and short-chain alkanols share a common channel site with only the bulkier long chain alcohols causing channel block. Thus, ethanol should compete with an inhibitory alcohol like octanol, but our preliminary studies are not entirely consistent with this prediction [30]–the increase in affinity induced by ethanol in a ten second flux assay was independent of the presence of a partially inhibiting concentration of octanol.

Finally, our data do not indicate whether ethanol exerts its effects on the nAcChoR at a saturable protein site or through a less specific mechanism. Since changes in membrane order parameters at 4°C were detectable at 500 mM, the lowest concentration studied [11], and the leftward shifting effect on agonist concentration-response curves appears not to be a saturable process, ethanol may be causing some sort of nonspecific membrane effect. However, the situation is complex for we observed little perturbation of function by ethanol in the absence of agonist.

Pharmacological perspectives

In order of decreasing sensitivity to ethanol four functions of the acetylcholine receptor were enhanced: agonist-induced fast desensitization; agonist-induced cation flux; agonist-induced slow desensitization, and ethanol-induced slow desensitization. The first two effects were the result of an increase in the apparent affinity of the agonist. Although this shift was equal for the two actions, the effect on fast desensitization, not previously reported, was greater in magnitude because it was accompanied by an increase in the maximum rate.

How large would these effects be at physiological concentrations? The anesthetic potency of ethanol, un-

like most general anesthetics, falls with decreasing temperature. Extrapolating data for tadpoles [11] to 4°C yields an anesthetic concentration of approx. 400 mM, while a value for frogs at 3°C is 330 mM [21]. Adopting a conservative value of 0.33 M, one may estimate that at low agonist concentration fast desensitization would be enhanced at least 6.1-fold, cation flux 4.2-fold and slow desensitization 1.6-fold. Even at threshold concentrations for inebriation (approx. one tenth the anesthetic concentration), fast desensitization would be enhanced 1.7-fold. At an intact synapse the net result would clearly depend on the degree of stimulation and its time course, but effects of this magnitude cannot be ignored in the etiology of ethanol's action. Indeed, their overall magnitude is not dissimilar from that recently reported for inhibition of NMDA-activated currents [31], suggesting that the role of central nicotinic receptors should also be considered.

Furthermore, the mechanism by which ethanol acts on the nAcChoR may be quite general. For example, the agonist concentration-response curves for the chloride flux mediated by the GABA receptor, a receptor which is homologous with the nAcChoR, is shifted to the left by ethanol [32]. The latter work also showed there were multiple effects of ethanol, for example desensitization was retarded. That ethanol exerts a multiplicity of actions on these targets suggests that there may be several underlying mechanisms at work. Currently it would be difficult to obtain the same degree of precision in GABA-stimulated chloride flux experiments as can be achieved in our preparation. Thus, a more detailed examination of ethanol's mechanisms of action at the nAcChoR may be the most efficient approach to elucidating the underlying mechanisms at work in this super-family. Such an approach may even be pertinent to such alcohol-sensitive channels as that activated by NMDA, where the inhibitory potency of alcohols is related to their lipid solubility [31].

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