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Ethanol Stabilizes the Open Channel State of the *Torpedo* Nicotinic Acetylcholine Receptor

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SUMMARY

Ethanol is known to cause a leftward shift of the acetylcholine concentration-response curve for channel opening of the nicotinic acetylcholine receptor (nAcChoR). However, it remains uncertain whether the mechanism underlying ethanol's effect is an increase in the binding affinity of the agonist to the receptor or an increase in the open/closed equilibrium for those receptors occupied by agonist. In the present study, this question was resolved by measuring the efflux of $^{86}Rb^+$ over 9 msec from *Torpedo* vesicles after rapid mixing with the partial agonist suberyldicholine with or without ethanol as appropriate. Suberyldicholine's concentration-response curve is bell-shaped. Two actions underlie this bell-shaped curve, namely activation at low concentration (apparent dissociation constant for activation, K_a = 38 μM) and self-inhibition at higher concentration (apparent dissociation constant for inhibition, K_b = 9 mM), but the overlap of these two actions only reduces the maximum observable flux by 20%. Increasing ethanol concentration from 0 to 0.9 M causes: a linear increase in the maximum response of the nAcChoR to suberyldicholine from 5 to 80% of the maximum induced by acetylcholine, a moderate increase in K_a, and no change in K_b. Analysis of our results using the sequential two-site binding model revealed that the main action of ethanol on nAcChoR was to increase the fraction of occupied receptors that open. The equilibrium constant describing this effect changed by 8-fold at anesthetic concentrations. Ethanol also decreased the affinity of suberyldicholine for its self-inhibition site by a comparable amount, suggesting that its main action is to stabilize the open state. In addition, ethanol caused a small increase in suberyldicholine's affinity for the agonist site.

Ethanol has been shown to enhance the action of cholinergic agonists at the nicotinic acetylcholine receptor by both electrophysiological studies and biochemical studies using rapid flux techniques (1–5). It promotes channel opening by acetylcholine (6), slows the decay rate of miniature endplate currents (3), and decreases the frequency of endplate power spectra, indicating that the rate of channel closing is slowed (7, 8). Rapid flux studies (for review see Refs. 9 and 10) show that ethanol causes a leftward shift of the acetylcholine concentration-response curve without changing the maximum response (2). These studies would seem to imply that the underlying change upon addition of ethanol lies in the binding step because the maximum flux at saturating concentrations of agonist remains unchanged. On the other hand, it was suggested that the opening probability of full agonists, such as acetylcholine and carbamylcholine, are so high that an additional increase in the presence of ethanol would be undetectable in practice (11). So it is not clear whether this leftward shift in the concentration-response curve in the presence of ethanol is caused by an increase in the affinity of the agonist for its receptor or by an increase in the open/closed equilibrium of the receptor itself.

One way of solving this problem would be to use agonists in which the ratio of opened to closed channels is lower. Such agonists belong to the class of partial agonists. However, partial agonism may arise from several other causes. For a ligand-gated ion channel like the nAcChoR, these include a low intrinsic open/closed equilibrium, a low cation conductivity, or the inhibition of the very channel that the agonist opens (12–14), an action qualitatively similar to that of local anesthetics (15). Previous studies show that a variety of agonists and partial agonists all open channels with the same conductivity in embryonic rat muscle cells (16). In many cases, the activating and the inhibitory actions of partial agonists overlap to such a degree that it is very difficult to study the agonist action independent of the inhibitory action. Therefore, it is important for our purposes to choose an agonist in which activation and inhibition are well separated. Given this condition, an agonist which also exhibits a low open/closed equilibrium would be ideal for our study.

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ABBREVIATIONS: nAcChoR, nicotinic acetylcholine receptor; α-BTX, alpha-bungarotoxin.

Among the available agonists, suberyldicholine has one of the highest affinities for channel opening in frog (17), mouse (18), and *Torpedo* (19). In the latter study, the apparent dissociation constants for activation and inhibition were separated by about 3 orders of magnitude, a very satisfactory situation for our purposes. More importantly, suberyldicholine had a substantially lower maximum response than the full agonists acetylcholine and carbamylcholine, and, thus, must have a lower open/closed equilibrium for nAcChoR in *Torpedo*. With such a partial agonist, we should be able to distinguish between the two alternate mechanisms hypothesized to underlie ethanol's action. First, if ethanol acts simply to increase binding affinity, suberyldicholine's apparent affinity for the nAcChoR will be increased, but the maximum flux it stimulates will only increase slightly as the small overlap of the activation and inhibitory curves is reduced. Second, if ethanol acts solely on the open/closed equilibrium, the apparent affinity will decrease and the maximum flux will increase until it has a value effectively equal to that of acetylcholine. A preliminary account of some of this work has appeared previously (20).

Experimental Procedures

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 by Braswell *et al.* (21). Membrane suspensions were kept frozen at −80°C and thawed within 48 hr before use. Before flux assays using acetylcholine or suberyldicholine, vesicles were incubated for 20 min with 0.1 mM diisopropylfluorophosphate at 4°C to inhibit acetylcholinesterase. Flux responses were not altered by this treatment (2).

Cation channel function was assayed by measuring agonist-induced $^{86}Rb^+$ efflux from spontaneously sealed native *Torpedo* electroplaque vesicles at 4°C. Vesicles were incubated with $^{86}Rb^+$ overnight and extravesicular radioactivity separated by exclusion and ion exchange chromatography. A rapid quenched-flow technique as described in detail in Forman *et al.* (2) was used for time-resolved studies. When necessary, the number of active receptor-channel complexes was reduced by blocking with the irreversible inhibitor, α-bungarotoxin (α-BTX) to prevent full equilibration of $^{86}Rb^+$ within the experimental assay time (19). Measurements of $^{86}Rb^+$ efflux from α-BTX-treated vesicles at 4°C were made at different periods after mixing with agonists, starting from the shortest time of 2–3 msec obtainable with the quenched flow apparatus. Flux times were calculated from measurement of flow rate through the rapid mixing device and the volume of the reaction tube used [the volume between the first mixer where flux is initiated and the second mixer where flux is quenched (19)] and calibrated by base hydrolysis of *o*-nitrophenyl acetate. Flux was quenched using 50 mM procaine. Ethanol was added to vesicles at the same time as the agonist at the first mixer. Thus, the concentration of both agents was elevated within about 100 μsec. After quenching at the second mixer, the reaction mixture was immediately filtered and the filtrate was sampled for scintillation counting. It is important to immediately filter the sample so that the time of nAcChoR exposure to ethanol was minimized. In practice, the total exposure time to ethanol was less than 5 sec and the background leak was unaffected by ethanol under these conditions.

The percentage of non-leak $^{86}Rb^+$ counts, F_A, is calculated according to the following equation:

$$F_A = \frac{\text{cpm(Ag, } t) - \text{cpm(Leak, } t)}{\text{cpm(Total)} - \text{cpm(Leak, } t)} \quad (1)$$

where cpm(Ag, t) are the counts per minutes of $^{86}Rb^+$ in the filtrate, cpm(Total) are those before filtration, and cpm(Leak, t) are the $^{86}Rb^+$ counts that leaked through the vesicles in the absence of agonist. Measurement of the cpm(Leak, t) at different times after the vesicles were eluted from the column revealed that it increases linearly during our typical 45-min experimental period. Hence, the leak correction was routinely made by measuring the cpm(Leak, t) after passage through the quenched flow apparatus at the beginning and the end of the experiment and a linear regression of cpm(Leak, t) versus time was made. Because residual agonist from the experiment may still be present in the mixer and it is hard to remove completely, 50 mM procaine was included in the final leak correction measurement to inhibit any residual agonist-induced flux. The procaine itself does not enhance the leak rate.

Initial $^{86}Rb^+$ efflux rates (k_f) were estimated according to Eq. 2:

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

where F_{eq} is the maximal flux signal possible in this system in the absence of α-BTX. The value of F_{eq} was measured for each batch of vesicles by exposing them to 10 mM acetylcholine for 10 sec. This equation corrects the response for the decline in the $^{86}Rb^+$ concentration gradient as the assay progresses (9).

The agonist concentration-response curve for suberyldicholine derived from flux measurements in *Torpedo* vesicles is bell-shaped and can be defined by the following biphasic logistic equation:

$$F_c = \text{Max(c)} * \frac{[A]^{n_a}}{[A]^{n_a} + K_a^{n_a}} * \left(1 - \frac{[A]^{n_b}}{[A]^{n_b} + K_b^{n_b}}\right) \quad (3)$$

It consists of two overlapping functions, one for activation and the other for inhibition. Max(c) is the maximum value of F_c if the activation and inhibition curves are separable, K_a is the agonist concentration producing 50% of the maximum response, n_a is the Hill coefficient for activation, K_b is the agonist concentration that inhibits 50% of maximum flux, and n_b is the Hill coefficient for self inhibition. Individual flux responses can be fitted by nonlinear least squares to Eq. 3.

Results

Two factors will affect the time course of the efflux of $^{86}Rb^+$ from nAcChoR vesicles after agonist binding to the receptor. One is fast desensitization of the nAcChoR in the presence of the agonist which happens at a rate of 5/sec (12). The other is the time scale upon opening the channel over which the concentration of $^{86}Rb^+$ inside the vesicles decreases toward the concentration outside to eventually achieve equilibrium. If our assay falls into these time domains, the consequent nonlinearity would prevent us from obtaining the flux rate accurately. To eliminate the effect of fast desensitization, the $^{86}Rb^+$ efflux from nAcChoR vesicles in response to acetylcholine and suberyldicholine must be measured over a sufficiently short time. To deal with the second effect, the efflux rate of $^{86}Rb^+$ can be decreased by reducing the number of active receptors in the vesicles with α-BTX blockade, so the vesicles are not emptied during the assay's integration time. The remaining decrease in ion gradient can be corrected using Eq. 2. In practice, this correction is small until about 30% of the concentration gradient has been discharged and becomes difficult to apply if more than 80% is discharged. Fig. 1 shows typical kinetic data for 10 mM acetylcholine- and 0.5 mM suberyldicholine-stimulated $^{86}Rb^+$ efflux from nAcChoR vesicles. For both agonists, the concentration used here stimulates the maximum $^{86}Rb^+$ efflux (see Ref. 2 for acetylcholine and Fig. 2 in this article for suberyldicholine). To correct for the discharge of the $^{86}Rb^+$ concentration gradient, F_c was obtained from Eq. 1 and plotted against time. The solid lines represent the initial rates in the beginning linear region in both cases. Suberyldicholine induces

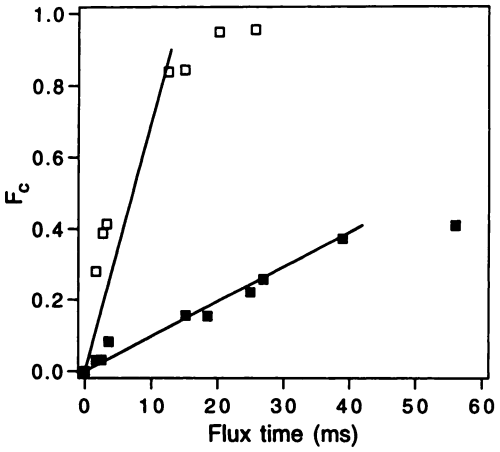


Fig. 1. Initial rates of $^{86}Rb^+$ efflux measured by quenched-flow at 4°C. Forty percent of nAcChoRs on the vesicles were blocked by α-BTX to ensure that the vesicles did not empty of $^{86}Rb^+$ during the assay. F_c values (□, 10 mM acetylcholine; ■, 0.5 mM suberyldicholine) were calculated according to Eq. 2 and plotted against the flux integration time. The solid lines were obtained by fixing the y-intercept through 0 and ignoring points beyond the linear kinetic region in both cases. The initial efflux rate for 10 mM acetylcholine is 270 ± 16 sec^{-1} and for 0.5 mM suberyldicholine is 25 ± 6.8 sec^{-1}.

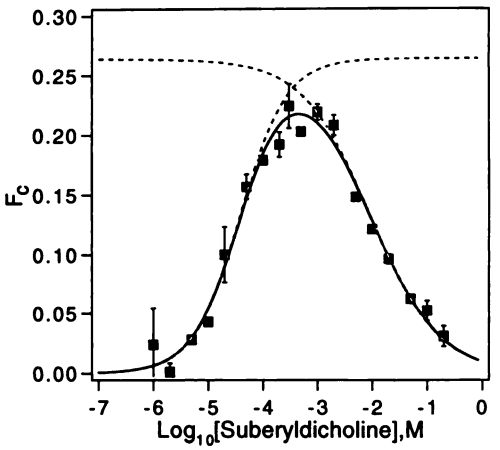


Fig. 2. A typical bell-shaped suberyldicholine concentration-response curve. The experiment was performed at 4°C with a 9-msec flux integration time. The nAcChoR vesicles were blocked with adequate amount of α-BTX to make the maximum flux, F_A, in the range of 0.2–0.3. In this particular experiment, the suberyldicholine concentration varied from 10^{-6} to 0.2 M. The *solid line* is obtained from fitting the data to Eq. 3. The following parameters, $n_a = 1.0 \pm 0.16$, $n_b = 0.69 \pm 0.12$, Max(c) = 0.26 ± 0.034, $K_a = 38 \pm 11$ μM, $K_b = 9 \pm 4.3$ mM were obtained from the fitting with no constraint. The *dashed lines* show the deconvolution of the underlying activation and inhibition curves according to Eq. 3.

an efflux of $^{86}Rb^+$ with an initial rate of 25 ± 6.8 sec^{-1}, much slower than that of acetylcholine, which has an initial rate of 270 ± 16 sec^{-1} under the same conditions in the identical batch of vesicles. Similar data had also been obtained previously (19). These allowed us to establish that an integration time of 10 msec or shorter falls well within the linear kinetic region when 10 mM acetylcholine is the agonist and that the comparable time for 0.5 mM suberyldicholine is 40 msec. Consequently, we chose to use 9 msec as the standard time of flux integration in the subsequent single-point kinetics quenched-flow studies for convenience.

Fig. 2 shows a typical curve of F_c against suberyldicholine concentration. As the concentration of suberyldicholine is raised above 1 μM, the flux response increases over a wide concentration range until a maximum is reached at about 0.5 mM. Increasing the suberyldicholine concentration further causes a decrease in flux response. Nonlinear least squares fitting with no constraint of the above data to Eq. 3 gives the following parameters: Max(c) = 0.26 ± 0.034, $n_a = 1.0 \pm 0.16$, $n_b = 0.69 \pm 0.12$, $K_a = 38 \pm 11$ μM, and $K_b = 9 \pm 4.3$ mM. The bell-shaped curve consists of two overlapping functions defined in Eq. 3, activation and inhibition, which are shown by the dashed lines in Fig. 2. Note that the calculated maximum for these dashed curves is about 20% higher than the envelope of the overlapping curves (solid line).

Only one such complete suberyldicholine concentration-response curve can be measured in one batch of $^{86}Rb^+$ loaded vesicles because of the time limits imposed by the leak problem, as mentioned in the Experimental Procedures section. Consequently, each suberyldicholine concentration-response curve at different ethanol concentrations was obtained from a different batch of vesicles. Because the fraction of blocked receptors in each batch of vesicles used in each experiment cannot be precisely controlled and small variations in α-BTX blockage have a big effect on the flux in the sensitive region (22), the relative maximum response at different ethanol concentrations cannot be obtained accurately by comparing individual curves. From Eq. 3, the shape of the concentration-response curve is independent of the maximum value, but is dependent on the n_a, n_b, apparent activation constant K_a, and apparent inhibition constant K_b. Hence, absolute values of n_a, n_b, K_a, and K_b can be obtained from Fig. 2 in each individual measurement regardless of the maximum response.

In order to obtain the relative maximum response at different ethanol concentrations, it is necessary to adopt some normalization scheme to compensate for the day-to-day experimental variations as well as for deliberate variation in the degree of α-BTX block. Therefore, we measured the apparent maximum flux response (without taking account of the overlap problem) to 0.5 mM suberyldicholine, which causes maximum stimulation, as a function of ethanol concentration in a single batch of vesicles and compared this with the maximum flux response to acetylcholine at 10 mM. The maximum flux stimulated by 10 mM acetylcholine, which is known to be unaffected by ethanol (2), is taken as unity. The resulting apparent maximum response at different ethanol concentrations is shown in Fig. 3. These data were from three separate experiments and the response at each ethanol concentration shown on the graph is an averaged value. The response of suberyldicholine relative to acetylcholine increases linearly from a control value of 0.05 ± 0.052 to a value of 0.68 ± 0.08 at 0.9 M ethanol.

Individual bell-shaped curves (such as that shown in Fig. 2) obtained at different ethanol concentrations with different batches of vesicles could be normalized to the averaged apparent maximum response value shown in Fig. 3 to minimize the variations between different batches of vesicles and different degree of blockage by α-BTX. The resulting bell-shaped curves at different ethanol concentrations with their maximum response relative to that of acetylcholine are displayed on a single graph in Fig. 4. The data points shown for each ethanol concentrtion are the average of three experiments and the flux at each suberyldicholine concentration in each experiment was measured two to four times. The collective data were then fitted to Eq. 3 by the nonlinear least squares procedure. Because there

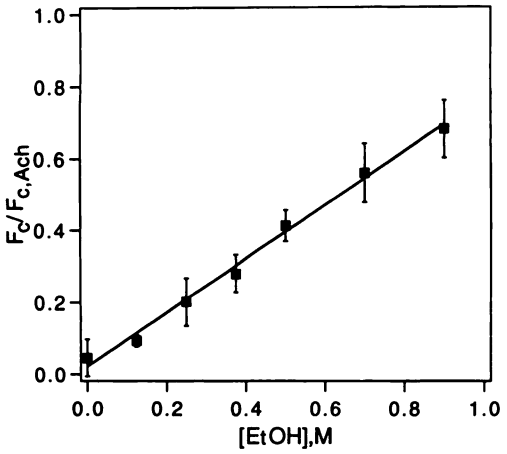


Fig. 3. The effect of increasing ethanol concentration on the maximum flux response to suberyldicholine. The experiment was performed at 4°C with a 9-msec flux integration time. The suberyldicholine concentration was fixed at 0.5 mM and the flux was measured at different ethanol concentrations from 0 to 0.9 M. The flux values of suberyldicholine in each experiment were normalized to the flux value at 10 mM acetylcholine for the same vesicles at 4°C with a 9-msec flux integration time. The data points are averages of three experiments and the error bars are the standard deviations of the data from all the three experiments at the same ethanol concentration. The solid line was obtained from the linear least squares fit to the data. The result of fitting is $y = (0.02 \pm 0.014) + (0.74 \pm 0.031)x$.

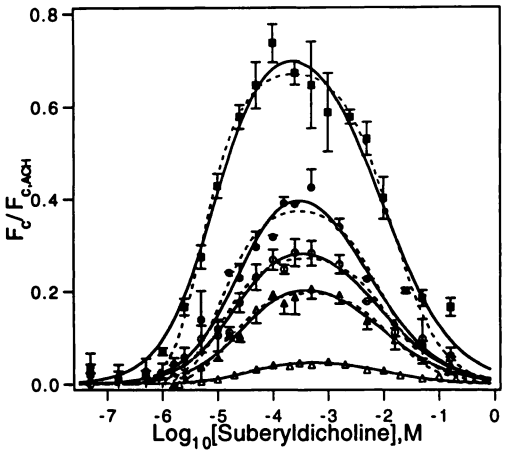


Fig. 4. Suberyldicholine concentration-response curves in the presence of various concentrations of ethanol. The experiments were done at 4°C with a 9 -msec flux integration time. Each suberyldicholine concentration-response curve was measured with the concentration of suberyldicholine varied from 5×10^{-6} M up to 0.15 M at a fixed ethanol concentration. The data at each suberyldicholine concentration are the mean values of three experiments in the presence of no ethanol (△), 0.25 M ethanol (▲), 0.38 M ethanol (○), 0.5 M ethanol (●), and 0.9 M ethanol (■). The *solid lines* were obtained from fitting the data at each ethanol concentration to Eq. 3 with nonlinear least squares regression with n_a and n_b fixed at 0.9 and 0.7, respectively. The fitting parameters are summarized in Table 1. The dashed lines were obtained by fitting to Eq. 4 (see Discussion).

are five parameters in Eq. 3 and the Hill coefficients are not expected to change at the concentrations of ethanol used here (2), fixing n_a and n_b would reduce the uncertainties in estimating the other three parameters. Examination of all the individual experiments with or without the presence of ethanol revealed that all of them have similar bell-shaped curves and have a mean n_a value of 0.9 ± 0.2 (standard derivation) and a mean n_b value of 0.7 ± 0.2. So it is reasonable to assume that all the curves have the same n_a and n_b, respectively. To be consistent with each measurement, n_a and n_b were fixed at 0.9 and 0.7, respectively, for all the ethanol concentrations during nonlinear least squares fitting to Eq. 3. The fitted parameters, K_a, K_b, and Max(c), are summarized in Table 1. It should be noted that the Max(c) value (we will refer it as actual maximum response) is the value without overlap of the activation and inhibition curves and, hence, should be higher than or equal to the experimental maximum response value. Over the ethanol concentration range studied (0–0.9 M), the actual maximum response, Max(c), changes more than 12-fold, whereas the apparent affinity for activation increases about 4.5-fold and the apparent affinity for inhibition remains constant.

Discussion

The low intrinsic open/closed equilibrium and adequate separation of the activation and inhibition curves of suberyldicholine make it an ideal agonist to study the mechanism of ethanol action on the nAcChoR. Fig. 2 shows a suberyldicholine concentration-response curve measured over a 9-msec integration time which exhibits a similar bell shape to that previously reported (19). A nonlinear least squares fit of the data shows that the apparent dissociation constant for activation (K_a) is 38 μM and for inhibition (K_b) is 9 mM. The observed maximum response (Fig. 2, *solid line*) is about 20% lower than the actual maximum response, Max(c) (Fig. 2, *dotted line*), because of a small degree of overlap between the activation and inhibition curves. It is clear for suberyldicholine that the overlap between the activation and inhibition curves only contributes slightly to its low efficacy, which therefore results mainly from its low intrinsic open/closed equilibrium. The advantage of suberyldicholine for the present study is that any increase in its intrinsic open/closed equilibrium or its binding affinity to the nAcChoR induced by ethanol can be assigned unequivocally.

Increasing ethanol concentrations from 0 to 0.9 M caused a linear increase in the apparent maximum flux induced by suberyldicholine from 0.05 to 0.68 relative to the maximum flux induced by acetylcholine (Fig. 3). However, a relatively smaller increase in K_a (about 4.5-fold) and no detectable change in K_b is observed (see Table 1 and Fig. 4). This shows that ethanol exerts its effect on the nAcChoR mainly by increasing the open/closed equilibrium when suberyldicholine is the agonist.

The action of ethanol on the suberyldicholine concentration-response curve observed here seems different from that of acetylcholine (2) and nicotine (5) in several aspects. At 1 M

TABLE 1

Parameters for suberyldicholine concentration-response curves at different ethanol concentrations

The parameters were obtained from the non-linear least squares fit to Equation 3 of the data shown in Figure 4. The Hill coefficients for activation (n_a) and inhibition (n_b) are fixed at 0.9 and 0.7, respectively (see text). The apparent dissociation constants of activation (K_a), inhibition (K_b) and the errors for both of them were obtained from the fitting. The actual maximum responses, Max(c), that occur at 0.5 mM suberyldicholine concentration at different ethanol concentrations were also obtained from the fitting in Figure 4, but their errors were obtained from Figure 3.

[EtOH]	Max(c)	K_a	K_b
M		μM	mM
0	0.06 ± 0.052	42 ± 7.8	9 ± 2.1
0.25	0.24 ± 0.067	24 ± 4.6	8 ± 2.3
0.38	0.34 ± 0.051	21 ± 3.5	8 ± 2.3
0.5	0.50 ± 0.043	26 ± 4.9	5 ± 1.6
0.9	0.79 ± 0.080	9 ± 1.2	9 ± 1.6

ethanol, K_a was reduced about 15 times when acetylcholine was the agonist. However, K_a was only reduced by 4.5 times in the presence of 0.9 M ethanol when suberyldicholine was the agonist. Although ethanol caused a leftward shift of the acetylcholine concentration-response curve without changing the maximum response, suberyldicholine caused the apparent maximum response to increase more than 12 times in the presence of 0.9 M ethanol. However, we cannot say just from inspection that ethanol only caused an increase of acetylcholine affinity to the receptor without changing its open/closed equilibrium. We will return to this point in a later section with a more detailed kinetic analysis.

The action of ethanol on the nicotine concentration-response curve is more complicated. Single-channel studies show that ethanol increases agonist affinity, probably by slowing its off rate, and does not affect conductance (6), but other studies suggest ethanol may slow channel closing (1, 23). These studies involve prolonged exposures to ethanol, and the ensuing desensitization is a complicating factor. It was recently shown that in the presence of sufficient amounts of ethanol, maximum stimulation by nicotine elicits essentially the same flux response as does a full agonist, and the difference between K_a and K_b is increased (5). The change of the efficacy of agonists at the nAcChoR can be due to a change in binding of the agonist or to a change in the open/closed equilibrium. Because of the significant overlap between nicotine's activation and inhibition curves, it is not certain whether only the affinity of nicotine for the nAcChoR is increased or whether both the affinity and maximum open/closed equilibrium are increased in the presence of ethanol.

To understand in more detail the basis of ethanol's action, we need to study the data with the aid of kinetic analysis. Agonists at the nAcChoR have been shown not only to activate, but also to inhibit the receptor ionophore complex (24). In single-channel studies on nAcChoR from frog neuromuscular junction, it could be seen that channel blocking events occur (25) and noise analysis of decamethonium's action at the nAcChoR showed two distinct components and a channel inhibition mechanism similar to that of procaine (15). These and similar observations have resulted in the classical sequential scheme of receptor activation being modified to include self-inhibition by the agonist itself. The following simplified model can be used to account for what happened in the 9-msec time domain when the agonist is rapidly mixed with the receptor. We assume that agonist (A) binds sequentially to two equivalent sites on the receptor (R) followed by channel opening (A_2R^o) and channel blocking (A_2R^*A) events in the following scheme.

$$A + R \underset{1/2K_1}{\rightleftharpoons} AR \overset{A}{\underset{2K_1}{\rightleftharpoons}} A_2R \underset{K_o}{\rightleftharpoons} A_2R^o \overset{A}{\underset{K_2}{\rightleftharpoons}} A_2R^*A$$

SCHEME 1

We define K_1 and K_2 as the dissociation constants for binding to the activation and inhibition sites, respectively, *i.e.*, $K_1=2[A][R]/[AR]=[A][AR]/2[A_2R]$ and $K_2=[A][A_2R^o]/[A_2R^*A]$, and for channel opening $K_o=[A_2R]/[A_2R^o]$. The following equation can be derived assuming, consistent with Fig. 1, that a steady state of open channels is maintained during our 9-msec assay,

$$\frac{[A_2R^o]}{[R_T]} = \frac{[A]^2}{\{^dK_2^2 \cdot K_o + 2[A] \cdot K_1 \cdot K_o + (1 + K_o + [A]/K_2]) \cdot [A]^2\}} \quad (4)$$

where $[R_T]$ is the total receptor concentration and $[A_2R^o]/[R_T]$ represents the overall open probability of the receptors when agonist is mixed with the receptor, which has been denoted as p_o in electrophysiological studies (11). It has been shown that at 10 mM acetylcholine, almost all the receptors are in the open state (11). Therefore, the concentration of receptors in the open state at 10 mM acetylcholine ($[A_2R^o]_{10mMAch}$) should be nearly equal to the total receptor concentration ($[R_T]$). In the present experiment, the measured overall opening probabilities were all normalized to that of 10 mM acetylcholine by assuming $[A_2R^o]/[R_T]=[A_2R^o]/[A_2R^o]_{10mMAch}$.

As depicted in the above scheme, the cation flux response to an agonist depends on the fraction of receptors in the open state (A_2R^o) and the conductance properties of the open ion channel. So there are several ways of interpreting our data within the framework of the sequential model. Ethanol could change the conductance of the channel while it is open. It might change the percentage of occupied receptors that are in the open state (decrease K_o). Or it could change the fraction of receptors that bind the agonist at a given concentration (decrease K_1). It is unlikely that ethanol changes the conductivity of the channel once the channel is open (1). Therefore, ethanol's mechanism of action is either to enhance the affinity of the agonist for the receptor, or to increase the open/closed equilibrium, or to reduce the overlap of K_a and K_b.

Some evidence based on both electrophysiological and rapid kinetic flux studies suggests that ethanol acts at the binding step by slowing the dissociation of the agonist (2, 6, 26). With full agonists, like acetylcholine, the open/closed equilibrium is already very high and any further increase may not be detectable (11). It thus remains in doubt whether this leftward shift in the concentration-response curve occurs because the binding affinity itself increases or because the open/closed equilibrium increases. For example, reanalyzing Forman's data (2) on the ethanol-induced leftward shift of acetylcholine concentration-response curves using the above theoretical model shows that either decreasing K_1 or decreasing K_o can result in a leftward shift of the concentration-response curve. Thus, in Fig. 5, an unconstrained nonlinear least squares fit (*solid lines*) of Forman's data to Eq. 4 yields the result that ethanol does not change K_o (around 0.04), but acts entirely by decreasing K_1 (from 312 μM to 21 μM with ethanol concentration increased from 0 to 1 M). However, the data may also be fit satisfactorily with the same equation, but with K_1 fixed at 312 μM and K_o decreasing from 0.039 to 2.8×10^{-4} (equivalent to open/closed equilibrium increasing) with increasing ethanol concentration from 0 to 1 M (dashed lines).

The two-site model can also be used to analyze the suberyldicholine results. Nonlinear least square fits of the data in Fig. 4 to Eq. 4 are shown by the dashed lines in the same figure. The parameters obtained from the fitting are summarized in Table 2. The results obtained by this model show that the major effect of increasing ethanol concentration is to increase the open/closed equilibrium, proportional to $1/K_o$, whereas K_1 decreases slightly and K_2 increases more markedly.

The results of the above analysis are summarized in Fig. 6, which shows the dependence on ethanol concentration of both

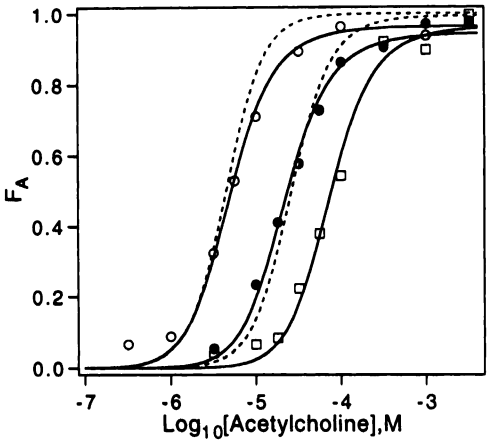


Fig. 5. Reanalysis of Forman's data (2) for ethanol's action on the acetylcholine concentration-response curve. The response (F_A) is plotted against acetylcholine concentration in the presence of no (□), 0.5 M (●), and 1 M (○) ethanol. The *solid lines* were calculated from nonlinear least squares fit to the data with no constraints using Eq. 4. Yielding parameters: K_1 = 312 ± 11 μM and K_o = 0.039 ± 0.021 (no EtOH); K_1 = 73 ± 12 μM and K_o = 0.039 ± 0.01 (0.5 M EtOH); K_1 = 21 ± 5.1 μM and K_o = 0.038 ± 0.01 (1 M EtOH). The *dashed lines* were obtained similarly, but with K_1 fixed at 312 μM. The parameters are: K_o = 5.6 ± 0.80 × 10^{-3} (0.5 M EtOH) and K_o = 2.8 ± 0.42 × 10^{-4} (1 M EtOH).

TABLE 2

Parameters obtained from fitting the suberyldicholine concentration response curves at different ethanol concentrations to the sequential model

The parameters were obtained from the non-linear least squares fit to Equation 4 of the data shown in Figure 4. The apparent dissociation constants of binding (K_1), channel opening (K_o), and channel blocking (K_2) and their errors were obtained from the fitting to Equation 4.

[EtOH]	K_o	K_1	K_2
M		μM	mM
0	19.1 ± 0.67	11 ± 1.6	0.7 ± 0.11
0.25	3.8 ± 0.16	9 ± 1.3	2.8 ± 0.52
0.38	2.5 ± 0.12	7 ± 1.0	3.7 ± 0.85
0.5	1.5 ± 0.10	8 ± 1.3	3.6 ± 0.87
0.9	0.44 ± 0.032	6.7 ± 0.80	9.6 ± 1.1

K_1, K_o, and K_2 for suberyldicholine and K_o for acetylcholine (with the assumption in the latter case that K_1 is fixed at 312 μM). For both agonists, a quantitatively self-consistent explanation is obtained where the main effect is a decrease in K_o with increasing ethanol concentrations whose magnitude is independent of agonist. This means that on a 9-msec time scale to a first approximation, ethanol acts solely by stabilizing the open state. It might be argued that because K_2 also depends on ethanol concentration, there should be an action on the inhibited state. However, K_2 changes largely as a result of the change in K_o (see Fig. 6 and Scheme 2). This can be demonstrated by the following argument in thermodynamic terms using the relationship $\Delta G° = -RT$.

If the action of ethanol is solely on the open state, then the standard free energy difference between the closed binding state (A_2R) and the blocked channel state (A_2R^*A) will be unchanged by ethanol (Scheme 2). Now the standard free energy difference between A_2R and the open channel state ($A_2R°$) is given by $\Delta G°_o = -RT\ln K_o$, and the standard free energy difference between $A_2R°$ and A_2R^*A is given by $\Delta G°_2 = -RT\ln K_2$. Therefore, the standard free energy difference between A_2R and A_2R^*A is obtained as $\Delta G°_o + \Delta G°_2 = -RT\ln K_o \cdot K_2$. The logarithm of the product $K_o \cdot K_2$ is plotted as a function of ethanol

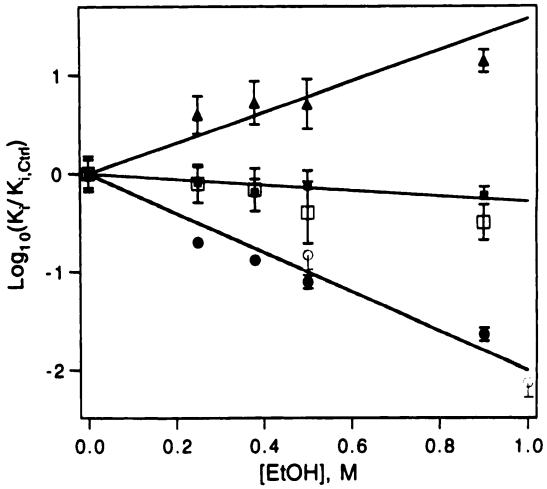
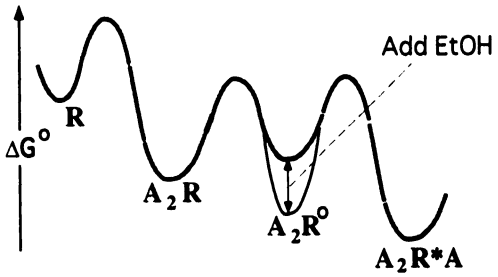


Fig. 6. Plot of $\log_{10}(K_i/K_{i,\,ctrl})$ at different ethanol concentrations with suberyldicholine as the agonist. K_i is the dissociation constant for K_o (●), K_1 (■), K_2 (▲), and $K_o K_2$ (□). $K_{i,\,ctrl}$ is the corresponding value in the absence of ethanol. The *solid lines* were drawn by linear least squares fitting through the origin. The fitted slope is −2.0 ± 0.14 (K_o), −0.29 ± 0.048 (K_1), and 1.6 ± 0.15 (K_2). The corresponding plot with acetylcholine as the agonist is also represented in the figure, where $K_{o,\,ach}$(○) is shown with K_1 fixed at 312 μM in all ethanol concentrations (see Fig. 5's legend and text). The *dashed lines*, which are obscured by the solid lines, were drawn by linear least squares fit to the acetylcholine data. The fitted slope for K_o is −2.0 ± 0.13.

concentration in Fig. 6 (*open squares*). It can be seen that it changes very little compared to K_o and K_2, which change reciprocally. The conclusion that can be drawn from the above analysis is that the major action of ethanol is to stabilize the open channel state relative to all the other states. An equivalent, but less probable explanation is that ethanol destabilizes the three other states equally. We can calculate the standard free energy changes in the $A_2R°$ state by $\Delta(\Delta G°) = (\Delta G°)_{no\ EtOH} - (\Delta G°)_{with\ EtOH} = RT\ln(K_o/K_{o,\,Ctrl}) = 5.3\ \log_{10}K$ ($KJ\cdot mol^{-1}$). From the slope in Fig. 6, which is $-2.0\ M^{-1}$ ethanol, it follows that the ethanol-induced standard free energy change in the open state $A_2R°$ is $-10.6\ KJ\cdot mol^{-1}\cdot M^{-1}$ of ethanol. It is not possible to assign the small remaining standard free energy change ($\sim -1.5\ KJ\cdot mol^{-1}\cdot M^{-1}$ of ethanol) to any state without arbitrary assumptions and we will not consider it further.

The following schematic free energy diagram can be drawn based on the above analysis, which clearly illustrates that the major action of ethanol is to stabilize the open channel state relative to all the other states.



SCHEME 2

It may be helpful to consider how the theoretical parameters in Table 2, which are obtained from the kinetic model may be related to the observed parameters in Table 1 which are obtained from the Hill equation. Because K_a is obtained from the

opening probability in the activation part of the concentration-response curve, it contains both the binding step (K_1) and the channel opening step (K_o) as seen from Scheme 1. The decrease in K_a is expected because it depends both on K_o, which decreases, and on K_1, which is relatively constant. That K_b is constant, whereas K_2 increases, appears paradoxical at first sight. However, this occurs because of the low fraction of open/closed channels. In the absence of ethanol, initially every channel inhibited by suberyldicholine can be replaced by one previously in the A_2R state. This buffering, which effectively increases the apparent K_b, becomes less important as the open/closed equilibrium is displaced toward the open state by increasing concentration of ethanol. Consequently, at high ethanol concentration, K_b and K_2 converge.

The present results clearly show that ethanol exerts its effects on nAcChoR mainly by increasing its open/closed equilibrium (lowering K_o value), and hence, stabilizing the open channel state. Even at the lowest ethanol concentration we have tested (250 mM), the increase in the open/closed equilibrium is dramatic (K_o decreased from 19.1 to 3.8). The anesthetic potency of most general anesthetics falls with decreasing temperature and our work was done at 4°C. Extrapolation of the ethanol data (27) for tadpoles to 4°C results in an anesthetic concentration of 400 mM. The anesthetic concentration of ethanol in frogs at 3°C is 330 mM. Extrapolation of our data for suberyldicholine-induced flux to a value of 350 mM ethanol shows a decrease in K_o of about 8-fold. Thus, the effect of ethanol at general anesthetic levels on suberyldicholine-induced flux is considerable. In fact, even at intoxicating concentrations, perhaps 10 to 20% of a general anesthetic level, K_o is decreased somewhat to ~16 to 14. It will be interesting to see if the neuronal nAcChoR exhibits a similar sensitivity. Although the effects we have measured occur at physiological concentrations, they are not shared by all general anesthetics and do not account for anesthesia per se.

The most significant conclusion of our work is that the acute action of ethanol on the nAcChoR is to selectively stabilize the receptor's open channel state in the physiological concentration range. What molecular feature of the nAcChoR is responsible for this stabilization is unclear. The linearity of Fig. 6 argues against a single binding site model, but interaction with a small fraction of a large number of low-affinity sites is not inconsistent with the data. Of the possible physical mechanisms, all of which would tend to be consistent with the observed linearity, those that act equally on forward and backward rates to alter activation energies; for example, lipid viscosity changes are not consistent with the data. However, because we did not resolve reaction rates, we cannot rule out that such effects occur in addition to those on the equilibrium constants. The location of ethanol's action could be anywhere on the nAcChoR that undergoes a structural change during the A_2R to A_2R^* conformation change, ranging from discrete locations such as the lumen of the channel to the larger regions involved in subunit-subunit interactions. We can exclude the unknown region where suberyldicholine exerts its inhibitory action because there is no major direct action on the inhibited state. It is clear that much work remains to resolve this problem.

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