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The Alkyl Chain Dependence of the Effect of Normal Alcohols on Agonist-induced Nicotinic Acetylcholine Receptor Desensitization Kinetics

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Background: **The nAcChoR is the prototypical member of a superfamily of ligand-gated ion channels that are all relevant targets of anesthetics and undergo desensitization upon prolonged exposure to agonist. This study was designed to investigate the effects of representative normal alcohols on the apparent rate of acetylcholine-induced nAcChoR desensitization.**

Methods: **Nicotinic acetylcholine receptors were obtained from the electroplax organ of *Torpedo nobiliana*. The apparent rate of acetylcholine-induced desensitization in the presence and absence of normal alcohols was measured using stopped-flow fluorescence.**

Results: **Normal alcohols as long as octanol (the longest studied) increased the apparent rate of desensitization induced by low concentrations of acetylcholine, shifting the agonist concentration–response curve for desensitization to the left. Ethanol, butanol, and, to a lesser extent, hexanol increased the maximal rate of desensitization induced by high, saturating concentrations of agonist. Beyond hexanol, heptanol and octanol had no effect on this maximal apparent rate of desensitization, even at concentrations that approach those that directly induce desensitization in the absence of agonist.**

Conclusion: **Normal alcohols ranging from ethanol to octanol increase the apparent affinity of nAcChoR for agonist with potencies that are proportional to their hydrophobicities. However, normal alcohol effects on the rate constant for desensitization show a cutoff beyond hexanol. This suggests that the effects of normal alcohols on the apparent agonist affinity and rate constant for desensitization of nAcChoR may be modulated by distinct sites that have different steric constraints; the site(s) responsible for increasing the maximal rate of desensitization are predicted to be smaller than those that increase the apparent agonist affinity. (Key words: Activation; anesthetic mechanism; cutoff; ion channel; *Torpedo.*)**

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THE nicotinic acetylcholine receptor (nAcChoR) is the prototypical member of a structurally and functionally related superfamily of ligand-gated ion channels that also includes the γ-aminobutyric $acid_A$, glycine, and 5-hydroxytryptamine$_3$ receptors.[1–3] Members of this superfamily play a critical role in neuronal synaptic transmission and are considered to be important targets of alcohols and other compounds that possess general anesthetic activity.[4] In particular, the nAcChoR from *Torpedo* is considered a useful model for studying the molecular mechanism of anesthetic action on ligand-gated ion channels because its structure and function are far better defined than any other superfamily member.[5–8] In fact, current structural and functional (kinetic) models of the other ligand-gated ion channels have been derived, in large part, by assuming similarity to the nAcChoR.

Electrophysiologic and ion flux studies have shown that the binding of two acetylcholine molecules to the nAcChoR rapidly leads to channel opening and ion flux.[9,10] However, upon prolonged exposure to agonist, the nAcChoR undergoes a slowly reversible transition to a desensitized conformational state that cannot be opened even by saturating concentrations of agonist.[10–12] This conformational transition, referred to as desensitization, is not unique to the nAcChoR because it has been observed in all other members of the ligand-gated ion channel superfamily, where it may represent an important mechanism by which synaptic transmission is modulated.[13–16]

Although normal alcohols (n-alcohols) are not used clinically, they have been widely used in mechanistic studies of anesthetic action because they form a homologous series that allows one to assess the role that molecular structure plays in defining anesthetic activity.[17–20] Such studies have shown that some of the actions of n-alcohols on ligand-gated ion channels vary

with their alkyl chain lengths, with potencies that are not simply proportional to their hydrophobicities. For example, rapid quench-flow studies indicate that although short-chain n-alcohols potentiate ion flux induced by low concentrations of agonist, n-alcohols longer than butanol do not.[21,22] Conversely, long-chain n-alcohols block ion flux through the nAcChoR open state, whereas short-chain n-alcohols do not.[21,23] Studies of the 5-hydroxytryptamine$_3$ receptor have shown that only short-chain n-alcohols potentiate ion flux induced by low agonist concentrations.[24] The inactivity of longer-chain members of a homologous series of n-alcohols is termed "cutoff" and has been cited as evidence of the existence of n-alcohol binding sites having limited dimensions on proteins.[25,26]

In this study, we quantitated the effects of representative n-alcohols on the apparent rate of nAcChoR desensitization induced by a wide range of acetylcholine concentrations to determine whether a cutoff is observed for anesthetic actions on nAcChoR desensitization kinetics. We used a stopped-flow fluorescence assay that measures desensitization rates even in the presence of n-alcohols that block ion flux. We determined that n-alcohols as long as octanol (the longest n-alcohol studied) increase the apparent rate of desensitization induced by low concentrations of acetylcholine, shifting the agonist concentration–response curve for desensitization to the left. This is indicative of an n-alcohol-induced increase in the apparent affinity of nAcChoR for agonist. Our studies also showed that ethanol, butanol, and, to a lesser extent, hexanol increased the rate of desensitization induced by high, saturating concentrations of agonist. However, beyond hexanol, we observed a cutoff in the ability of n-alcohols to increase the maximal rate of desensitization. Our results are discussed in terms of current kinetic models that describe the actions of anesthetic compounds on nAcChoR conformational transitions.

Materials

Torpedo nobiliana was obtained from Biofish Associates (Georgetown, MA). Diisopropylfluorophosphate, acetylcholine, carbamylcholine, and n-alcohols were purchased from Sigma Chemical Co. (St. Louis, MO). The fluorescent partial agonist, [1-(5-dimethylaminonaphthalene)sulfonamido] *n*-hexanoic acid β-(*N*-trimethylammonium bromide) ethyl ester (Dns-C_6-Cho), was synthesized according to the procedure of Waksman *et al.*[27]

Methods

Preparation and Characterization of nAcChoR-rich Membranes

Receptor membranes were obtained from freshly dissected *Torpedo nobiliana* electric organs and prepared using sucrose density gradient centrifugation, essentially as described by Braswell *et al.*[28] Membranes were stored in *Torpedo* physiologic solution (250 mM NaCl, 5 mM KCl, 3 mM CaCl, 2 mM $MgCl_2$, 5 mM NaH_2PO_4, and 0.02% NaN_3, *p*H 7.0) at −80°C and thawed on the day of use. Acetylcholinesterase activity was inhibited by exposing membranes to 3.0 mM diisopropylfluorophosphate for 30 min before dilution to the desired receptor concentration. The number of agonist binding sites was determined from Dns-C_6-Cho titrations.[29]

Determination of the Apparent Rate of Agonist-induced Desensitization

The apparent rate of agonist-induced desensitization was determined with a double-agonist pulse assay using a sequential mixing stopped-flow spectrofluorometer (Applied Photophysics, Leatherhead, England) as previously described.[30] In brief, receptor-rich membranes were first preincubated with acetylcholine for periods ranging from 15 ms to several minutes. The number of receptors able to be activated (nondesensitized) that remained after this preincubation period was then quantitated from the amplitude of the rapid fluorescence signal observed when the membrane/acetylcholine solution was rapidly mixed with the fluorescent partial agonist Dns-C_6-Cho and high, channel-activating concentrations of acetylcholine. This rapid fluorescence signal reflects the binding of Dns-C_6-Cho to the agonist self-inhibitory site on open state receptors and the conformational transition that follows.[29] In each experiment, receptor membranes (0.8 μM agonist binding sites) were loaded into one of the premix syringes of the spectrofluorometer, and acetylcholine was loaded into the other premix syringe. The solutions were rapidly mixed (1 ms mixing time; 1:1, vol:vol) and allowed to preincubate for the desired time. The nAcChoR/acetylcholine solution was then mixed (1 ms mixing time; 1:1, vol:vol) with a solution containing 10 mM acetylcholine and 20 μM Dns-C_6-Cho, and the fluorescence emission was recorded. Where appropriate, all of these solutions also contained the desired n-alcohol. An excitation wavelength of 290 nm was provided by a 150-watt xenon arc lamp, and the monochromator bandpass was 5 nm. Fluorescence emission greater than 500 nm was measured through a high

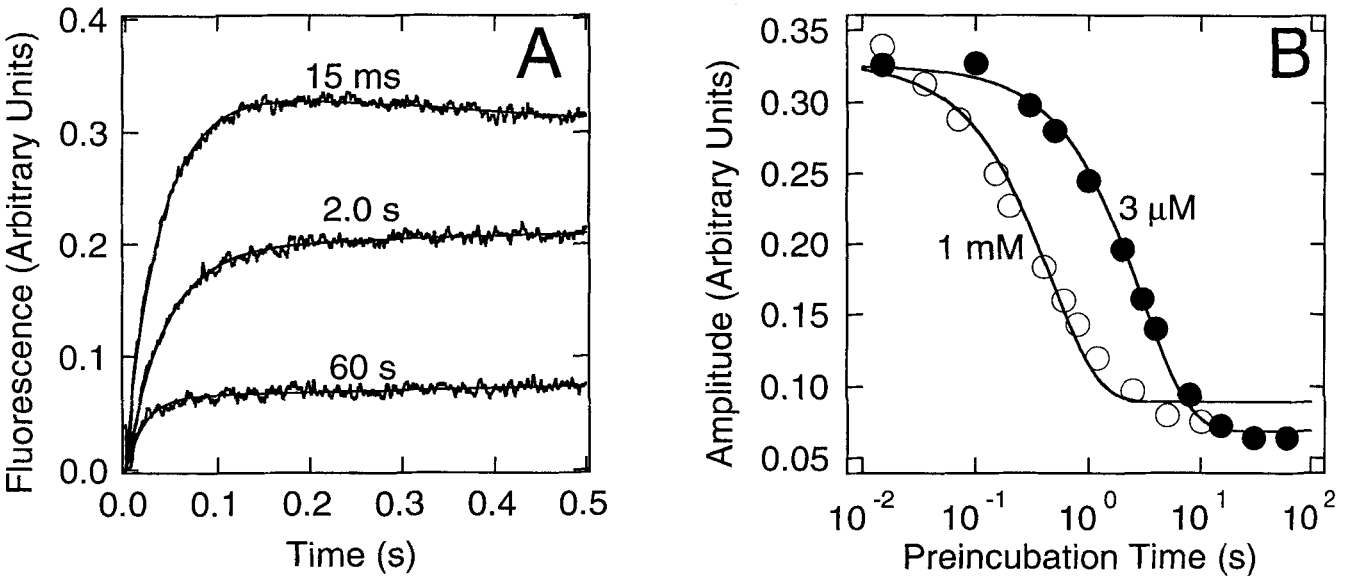


Fig. 1. The effect of acetylcholine-induced desensitization on the amplitude of the fast fluorescence component. (*A*) Fluorescence traces obtained when nAcChoR membranes are preincubated with 3 μM acetylcholine (final concentrations after mixing) for the indicated times and then rapidly mixed with 5 mM acetylcholine and 10 μM Dns-C_6-Cho (final concentrations). The amplitude of the fast fluorescent component was 0.341, 0.197, and 0.064 (arbitrary units) upon preincubation for 15 ms, 2 s, and 60 s, respectively. (*B*) The amplitude of the fast fluorescent component as a function of preincubation time is plotted using preincubating acetylcholine concentrations of either 3 μM or 1 mM. The curves are fits of the data to an exponential equation to derive the apparent rates of acetylcholine-induced desensitization. Acetylcholine at concentrations of 3 μM and 1 mM induced desensitization with apparent rates of 0.34 ± 0.02 s^{-1} and 2.2 ± 0.3 s^{-1}, respectively.

pass filter. Fluorescence intensity was recorded for 500 ms after the second mixing step. In a typical experiment, three to five individual runs were signal averaged to reduce noise. All experiments were performed at 20 ± 0.3°C.

Statistical Analysis

Experiments were performed using five separate preparations, and the effects of each n-alcohol were studied using at least two preparations. Data points on agonist concentration–apparent desensitization rate curves were fit to a Hill equation in the form:

$$k_{app} = k_{max} \cdot \left(\frac{[\text{Agonist}]^n}{[\text{Agonist}]^n + (K_d^{app})^n} \right) \quad (1)$$

where k_{app} is the experimentally determined apparent rate of desensitization, k_{max} is the maximum apparent rate of desensitization induced by high agonist concentrations, k_d^{app} is the agonist's apparent K_d for desensitization, and n is the Hill coefficient. The reported errors for the fitted parameters are the standard deviations derived from the curve fit.

Results

Figure 1A shows fluorescence traces from a series of sequential mixing stopped-flow experiments in which nAcChoR-rich membranes were preincubated with 3 μM acetylcholine for the indicated times and then rapidly mixed with *Torpedo* physiologic solution containing 5 mM acetylcholine and 10 μM Dns-C_6-Cho (final concentrations after mixing). When nAcChoR membranes were preincubated with acetylcholine for periods that are too brief to allow significant desensitization to occur (*i.e.*, 15 ms), there was a large, approximately exponential increase in fluorescence on the time scale of tens of milliseconds upon mixing with the acetylcholine/Dns-C_6-Cho solution. Nonrandom residuals indicated that there was also a slow linear fluorescence component. However, this linear component typically represented less than 10% of the trace and, therefore, was not analyzed in detail. Least-squares fitting of the entire fluorescence trace to an exponential equation with a linear component yielded an amplitude of 0.341 (arbitrary units) for the rapid fluorescent component (fig. 1). In experiments using longer preincubation times that permitted significant acetylcholine-induced nAcChoR desensitization to

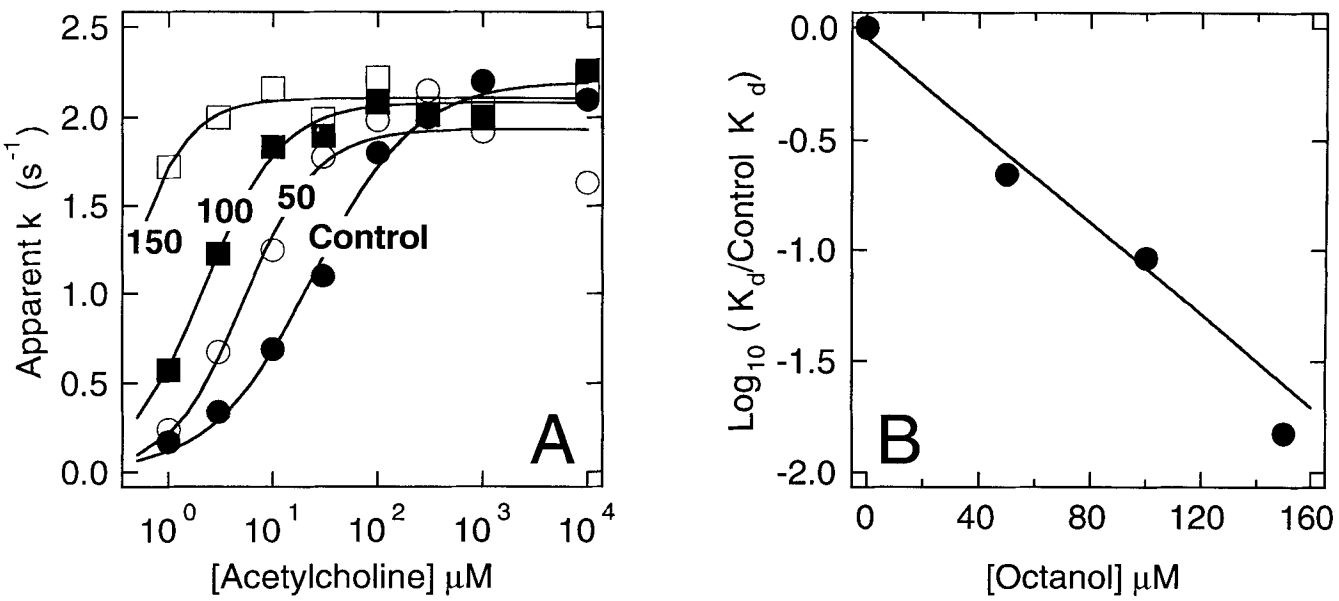


Fig. 2. The effect of octanol on acetylcholine concentration–response curves for desensitization. (*A*) The apparent rate of desensitization *versus* acetylcholine concentration is plotted using membranes that had been equilibrated with the indicated concentrations of octanol (in micromoles). The solid lines are fits of the apparent rate data to equation 1. Preequilibrating membranes with 50, 100, or 150 μM octanol reduced the apparent K_d for desensitization to 5 ± 1 μM, 2.2 ± 0.3 μM, or 0.4 ± 0.3 μM, respectively, from a control value of 24 ± 4 μM. The Hill coefficients ranged from 0.9 to 1.5, and the maximal rate of desensitization was approximately 2. (*B*) The logarithm of the normalized K_d is plotted as a function of octanol concentration. The solid line is a linear least-squares fit to the data obtained with 0, 50, and 100 μM octanol. The 150-μM point was not included in the fit because of the large uncertainty in its value. The slope of the line is −0.010 ± 0.002 s^{-1} mM^{-1}.

occur before mixing with the Dns-C_6-Cho/acetylcholine assay solution, the amplitude of the rapid fluorescence signal was smaller. Figure 1B plots the amplitude of this fluorescence signal as a function of acetylcholine-nAcChoR preincubation time using acetylcholine concentrations of either 3 μM or 1 mM during the preincubation period. The amplitude of the rapid fluorescence signal decreased exponentially with preincubation time, reflecting the process of agonist-induced desensitization. A fit of this amplitude data yielded apparent desensitization rates of 0.34 ± 0.02 s^{-1} or 2.2 ± 0.3 s^{-1} upon preincubation with 3 μM or 1 mM acetylcholine, respectively.

Figure 2A shows the effect of octanol on the apparent rate of desensitization induced by preincubation with a range of acetylcholine concentrations. Both in the presence and absence of octanol, the apparent rate increased with acetylcholine concentration before reaching a plateau at high acetylcholine concentrations. At each octanol concentration, a plot of the apparent rate versus acetylcholine concentration was fit to equation 1. In the absence of octanol, this membrane preparation showed an apparent K_d for acetylcholine of 24 ± 4 μM, a maximal rate of desensitization of 2.2 ± 0.1 s^{-1}, and a Hill coefficient of 0.9 ± 0.1. Octanol increased the apparent rate of desensitization induced by low concentrations of acetylcholine but had no significant effect on the apparent rate induced by high concentrations of acetylcholine. This resulted in a leftward shift in the acetylcholine concentration-response curve for desensitization that was octanol concentration-dependent. In the presence of 50 μM octanol, the apparent K_d of acetylcholine was reduced to 5 ± 1 μM. By 150 μM, octanol reduced the apparent K_d for acetylcholine to less than 1 μM, the lowest acetylcholine concentration of agonist used in the assay. We did not determine the effect of octanol at concentrations greater than 150 μM because at such high concentrations, octanol itself desensitized a significant fraction of the nAcChoRs, even in the absence of agonist. This was detected in experiments using a 15-ms preincubation period as a significant reduction in the amplitude of the rapid fluorescence signal in the presence of high octanol concentrations relative to that in the absence of octanol (data not shown). Figure 2B shows a plot of the logarithm of the normalized apparent K_d of acetylcholine as a function of octanol concentration and demonstrates that the apparent K_d of acetylcholine decreased approximately logarithmically with octa-

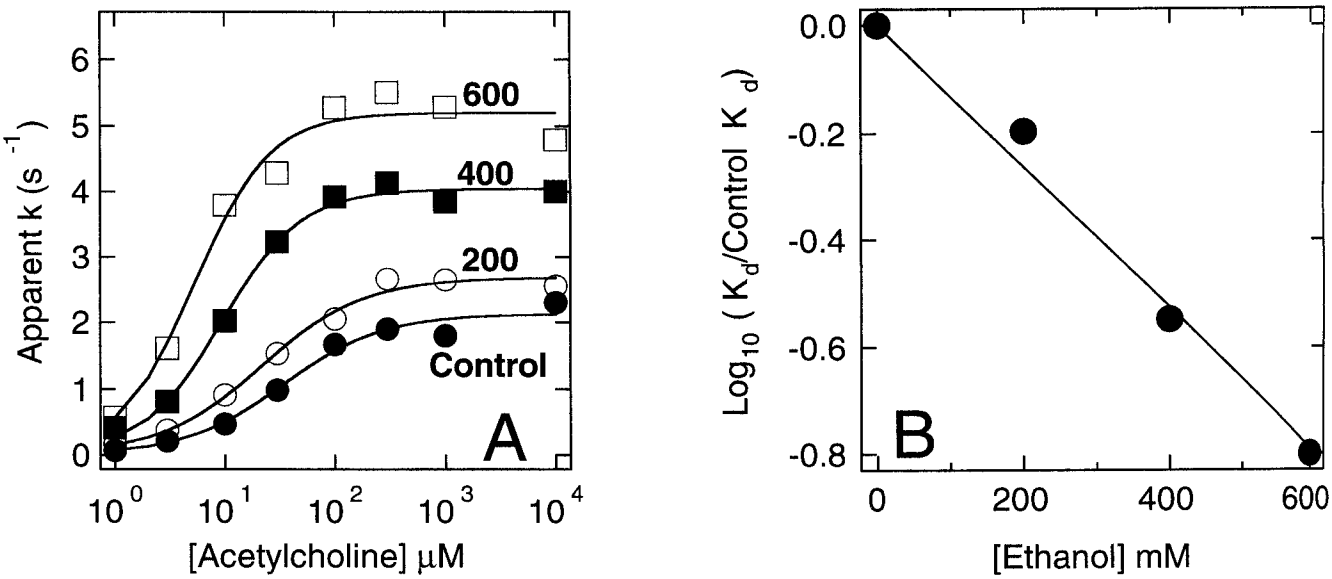


Fig. 3. The effect of ethanol on acetylcholine concentration–response curves for desensitization. (*A*) The apparent rate of desensitization *versus* acetylcholine concentration is plotted using membranes that had been equilibrated with the indicated concentrations of ethanol (in micromoles). The solid lines are fits of the apparent rate data to equation 1. Preequilibrating membranes with 200, 400, or 600 mM ethanol reduced the apparent K_d for desensitization to 21 ± 3 μM, 9.5 ± 0.9 μM, or 5.3 ± 0.9 μM, respectively, from a control value of 34 ± 8 μM. The Hill coefficients ranged from 0.9 to 1.3. (*B*) The logarithm of the normalized apparent K_d is plotted as a function of ethanol concentration. The solid line is a linear least-squares fit to the data with a slope of −0.0014 ± 0.0001 mM^{-1}.

nol concentration. A linear fit of this data up to 100 μM yielded a slope of −0.010 ± 0.002/μM. Forman *et al.*[31] previously quantified the reduction in the apparent K_d for ion flux induced by short-chain n-alcohols by defining a parameter, SC_{50}, which is equal to the n-alcohol concentration that reduces the apparent K_d of acetylcholine for ion flux by half. In their studies, the apparent K_d also decreased logarithmically with ethanol concentration. We used an analogous treatment to the desensitization data in figure 2B and determined that the SC_{50} of octanol for desensitization (the concentration of octanol that reduces the apparent K_d for desensitization by half) was 33 ± 7 μM.

Figure 3A shows the effect of ethanol on acetylcholine concentration–response curves. Ethanol had two effects on acetylcholine-induced desensitization. As with octanol, ethanol reduced the apparent K_d of acetylcholine for desensitization in a concentration-dependent manner. By 600 mM ethanol, the highest concentration studied, the apparent K_d was reduced from a control value of 34 ± 8 μM to 5.3 ± 0.9 μM. Figure 3B plots the logarithm of the normalized apparent K_d of acetylcholine as a function of ethanol concentration. A linear fit of this data yielded a slope of −0.0014 ± 0.0001 mM^{-1} and, therefore, an SC_{50} for desensitization of 220 ± 15 mM. In addition to reducing the apparent K_d of acetylcholine, ethanol also significantly increased the maximal apparent desensitization rate induced by high acetylcholine concentrations. This maximal rate increased approximately linearly, with ethanol concentration from 2.1 ± 0.1 s^{-1} (control) to 5.2 ± 0.4 s^{-1} by 600 mM. The potency with which ethanol increased this rate was quantitated as the slope of a linear fit of a plot of the maximal apparent rate (k_{max} in equation 1) *versus* ethanol concentration (fig. 4). This fit indicated that the maximal apparent rate of desensitization increased by 0.0053 ± 0.0006 s^{-1} for each millimole of ethanol, corresponding to a 48% increase in the maximal apparent rate at a concentration equal to the EC_{50} of ethanol for anesthesia in tadpoles (190 mM).

Butanol and hexanol behaved in a manner that was similar to ethanol in that they both reduced the apparent K_d of acetylcholine and increased the maximal apparent rate of desensitization (table 1). However, when normalized to its SC_{50} or *in vivo* anesthetic EC_{50}, it is apparent that hexanol increased the maximal apparent rate to a much lesser extent than either ethanol or butanol. Although heptanol substantially reduced the apparent K_d of acetylcholine, it did not increase the maximal apparent rate of desensitization, even at concentrations as high as 800 μM. A plot of the maximal apparent rate of desensitization as a function of heptanol concentration

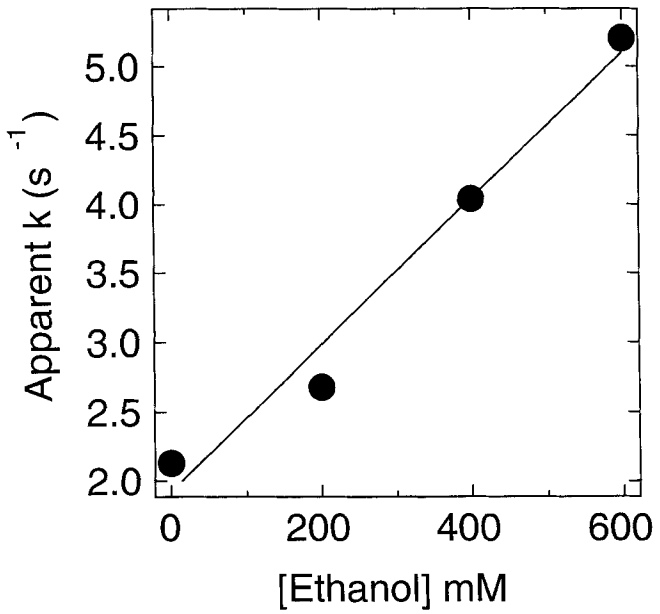


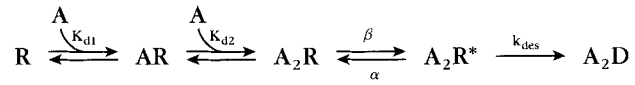
Fig. 4. The effect of ethanol on the maximal apparent rate at high acetylcholine concentrations. The maximal apparent rate increased linearly with ethanol concentration to a value of 5.2 ± 0.4 s^{-1} by 600 mM. The line is a linear least-squares fit of the data yielding a slope of .0053 ± 0.0006 s^{-1} mM^{-1}.

had a slope that was not significantly different from zero (table 1). At concentrations greater than 800 μM heptanol, we could not reliably measure the apparent rate of acetylcholine-induced desensitization because heptanol itself desensitized a significant fraction of all receptors.

Discussion

To our knowledge, this is the first study to define the alkyl chain-length dependence of the effects n-alcohols on the kinetics of agonist-induced desensitization of a ligand-gated ion channel. Our study demonstrates that n-alcohols as long as octanol increase the apparent rate of nAcChoR desensitization induced by low concentrations of acetylcholine. The potencies with which n-alcohols act increases logarithmically with the addition of successive methylene groups and in proportion to their hydrophobicities and *in vivo* anesthetic potencies.[32] Our study also shows that short-chain n-alcohols increase the maximal rate of desensitization induced by high, receptor-saturating concentrations of agonist, whereas long-chain n-alcohols do not.

The effects of n-alcohols on the kinetics of agonist-induced nAcChoR desensitization may be considered within the framework of a relatively simple scheme describing the processes of agonist binding, channel opening, and receptor desensitization:



where R, AR, and A_2R are the unliganded, monoliganded, and doubly liganded (closed) resting states; A_2R^* is the ion permeable, open state; and A_2D is the desensitized state. The rate constant for resensitization of A_2D back to A_2R^* has been omitted because it is six orders of magnitude slower than the rate constant for desensitization.[29] The binding of two agonist molecules to unliganded resting state nAcChoRs rapidly leads to channel opening followed by desensitization. This scheme predicts that the apparent rate of desensitization will increase with agonist concentration before reaching a plateau at a value equal to the rate constant for desensitization (k_{des}). Previous ion flux studies have estimated the rate constant for desensitization to be in the range of 2–7 s^{-1}.[33–35]

Rapid quenched-flow studies have shown that ethanol and butanol reduce the apparent K_d of acetylcholine for ion flux. The concentrations of ethanol and butanol that reduce the apparent K_d of acetylcholine for ion flux by half are 270 mM and 17 mM, respectively.[21] Our study shows that 220 mM ethanol and 16 mM butanol reduce the apparent K_d of acetylcholine for desensitization by half (table 1). The most economical explanation for the near identity in n-alcohol potency for enhancing agonist-induced ion flux and desensitization is that n-alcohols reduce the apparent K_ds for both channel activation and desensitization by acting at a common kinetic step(s), and that the primary pathway to the desensitized state in the presence of these n-alcohols remains *via* the doubly liganded open-channel state as depicted in scheme 1. The effect of ethanol and butanol on the apparent K_ds of acetylcholine for ion flux and desensitization may reflect an increase in either the microscopic affinity for agonist or the open/closed equilibrium (β/α). Our data do not allow us to distinguish between these two possibilities; however, quenched-flow studies indicate that ethanol increases the apparent agonist affinity for channel activation primarily by modifying the equilibrium between open and closed states.[36] More specifically, single-channel studies using nAcChoRs expressed in BC3-H1 cells suggest that butanol increases the opening rate constant, β.[20] From the onset of the current response to the rapid application of acetylcholine, Liu *et al.*[20] concluded that

Table 1. Effect of n-Alcohols on the Apparent K_D of Acetylcholine and the Maximal Rate of Acetylcholine-induced Desensitization

n-Alcohol	EC_{50} for Anesthesia* (mM)	SC_{50}† (mM)	Increase in k_{max}‡ (s^{-1} mM^{-1})	Increase in k_{max} at the SC_{50} Concentration§ (s^{-1})	Concentrations Studied (mM)
Ethanol	190 ± 41	220 ± 15	0.0053 ± 0.0006	1.2	200, 400, 600
Butanol	10.8 ± 0.77	16 ± 1	0.09 ± 0.01	1.4	10, 20, 40
Hexanol	0.57 ± 0.037	0.34 ± 0.07	0.66 ± 0.08	0.22	0.5, 1.0, 1.5
Heptanol	0.23 ± 0.011	0.15 ± 0.03	0.4 ± 0.4	0.06	0.3, 0.4, 0.6, 0.8
Octanol	0.057 ± 0.0025	0.033 ± 0.007	−1 ± 1	−0.03	0.05, 0.1, 0.15

* Righting reflex in tadpoles from Alifimoff *et al.*[17]

† Concentration of n-alcohol that reduces the apparent K_D for desensitization by half. The SC_{50} for each n-alcohol was derived from a linear fit of a plot of the logarithm of the apparent K_D *versus* n-alcohol concentration. The errors were obtained from the fit.

‡ Slope of a plot of the maximal rate of desensitization *versus* n-alcohol concentration. For each n-alcohol, the slope was derived from a linear fit of a plot of the maximal rate of desensitization from equation 1 *versus* n-alcohol concentration. The errors were obtained from the fit.

§ Equal to $k_{max} \times SC_{50}$ concentration.

20 mM butanol increased β by approximately twofold. Within the context of scheme 1, this action alone will quantitatively account for the twofold reduction in the apparent K_ds of acetylcholine for desensitization and ion flux, as well as the doubling of the single-channel burst frequency in the presence of low concentrations of acetylcholine induced by 20 mM butanol.

In view of previous rapid quenched-flow studies indicating that n-alcohols longer than butanol have no effect on the apparent agonist affinity of the nAcChoR, we were surprised to observe that long-chain n-alcohols significantly reduced the apparent K_d of acetylcholine for desensitization. One possible explanation for this apparent discrepancy is that long-chain n-alcohols potentiate agonist-induced desensitization *via* kinetic pathways not described by scheme 1 (*i.e.*, that do not pass through the open-channel state). However, because the potencies with which long-chain n-alcohols reduce the apparent K_d of acetylcholine for desensitization may be reasonably predicted simply by extrapolating the potencies of short-chain n-alcohols (potency increases smoothly in a logarithmic manner with alkyl chain length upon ascending the series from ethanol to octanol), it seems unlikely that the underlying kinetic mechanism by which short- and long-chain n-alcohols alter the apparent affinity of acetylcholine is different. Alternatively, quenched-flow studies use a protocol in which agonist and n-alcohols are added simultaneously to receptor membranes to minimize n-alcohol-induced passive ion leak across synaptosomes. Conceivably, the simultaneous addition of agonist and n-alcohol may not allow long-chain n-alcohols, which are present at micromolar concentrations, to fully equilibrate with their sites of action and exert their maximal potentiating effects on the time frame of the assay. In addition, any increase in the apparent agonist affinity of nAcChoR may be difficult to detect in ion flux studies because these n-alcohols also block flux through open channels. In support of this suggestion, when Liu *et al.*[20] used nAcChoRs that had been equilibrated with n-alcohols, they observed that n-alcohols as long as decanol increased single-channel burst frequency in the presence of 0.2 μM acetylcholine and concluded that this was consistent with an increase in the apparent affinity of acetylcholine for nAcChoR activation. In fact, figure 5 demonstrates that the con-

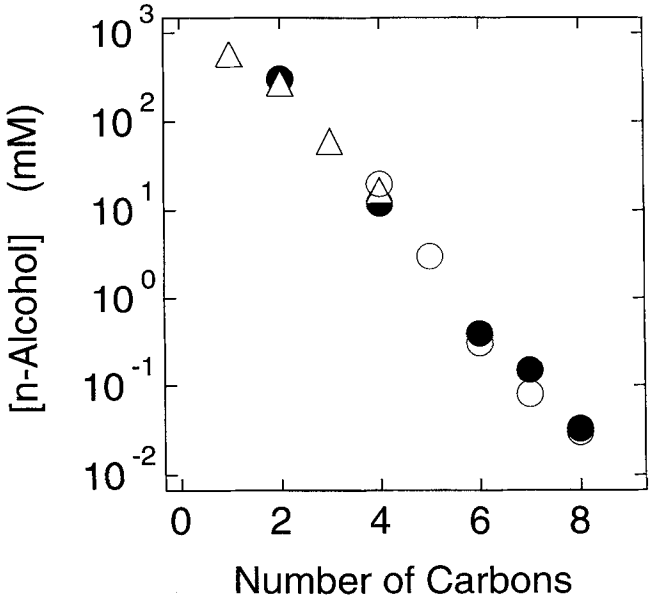


Fig. 5. Alkyl chain-length dependence of the concentration of n-alcohol that (1) reduces the apparent K_d of acetylcholine for desensitization by half (●), (2) reduces the apparent K_d of acetylcholine for ion flux by half (△),[21] and (3) doubles the frequency of bursts in single-channel recording of nAcChoRs in the presence of 0.2 μM acetylcholine (○).[20]

centrations of n-alcohols that double the single-channel burst frequency of nAcChoR in the presence of 0.2 μM acetylcholine agree closely with our SC_{50} values for desensitization, strongly suggesting that desensitization proceeds primarily *via* the open (albeit n-alcohol-blocked) state even in the presence of long-chain n-alcohols.

It has been reported, and our study confirms, that ethanol increases the maximal apparent rate of desensitization induced by saturating concentrations of agonist.[31] Within the context of scheme 1, this reflects an increase in the rate constant for desensitization (k_{des}). Our study shows that butanol and hexanol also increase the rate constant for desensitization. Of note, when each n-alcohol is normalized to its SC_{50}, hexanol is noted to be significantly less potent than either ethanol or butanol (table 1). The potency with which these n-alcohols enhance this rate constant increases approximately logarithmically with alkyl chain length. From this logarithmic trend, we would predict that heptanol and octanol should increase the rate constant by 2.5 s^{-1} mM^{-1} and 8.5 s^{-1} mM^{-1}, respectively. However, neither of these n-alcohols increased this rate constant, even at concentrations that (1) are predicted to do so by extrapolation of the potencies of shorter-chain n-alcohols, and (2) cause a large decrease in the apparent affinity of acetylcholine. In fact, even concentrations of heptanol and octanol that approach those that directly desensitize receptors (in the absence of agonist) produced no increase in the rate constant for desensitization.

Although our functional studies do not address specifically the question of whether the effects of n-alcohols on desensitization kinetics reflect a primary protein or lipid site of action, it has been suggested that cutoff may reflect the inability of long-chain n-alcohols to fit completely within a hydrophobic protein pocket.[25,37] Within the context of such theories, the observation that the n-alcohol effect on the rate constant for desensitization shows cutoff between hexanol and heptanol, whereas the effect on the apparent agonist affinity continues at least to octanol, suggests that these two kinetically distinct effects on nAcChoR may be modulated by physically distinct sites that have different steric constraints; the site(s) responsible for increasing the rate constant for desensitization are predicted to be smaller than those that increase agonist affinity. Although the locations of such protein sites have not yet been defined, it is tempting to speculate that the smaller sites might be located within the confined spaces between hydrophobic membrane-spanning domains, whereas larger sites might be at the protein-lipid or protein-water interfaces. Ultimately, studies that use n-alcohols that are able to be photoactivated and conformationally restricted cyclic alcohols may help to define both the location and dimensions of putative receptor sites.

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