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Short Chain and Long Chain Alkanols Have Different Sites of Action on Nicotinic Acetylcholine Receptor Channels from *Torpedo*

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SUMMARY

At nicotinic acetylcholine receptors, short chain *n*-alcohols (alkanols) have excitatory actions, whereas long chain alkanols inhibit channel activity. This study tests a previously proposed unitary hypothesis that suggests that these contrasting actions can be accounted for by interaction at just one hydrophobic site within the ion channel lumen. All alkanols bind to this site, but only long chain alkanols are large enough to completely block the channel. Short chain alkanols are too small to cause any channel occlusion, and in binding to the site they stabilize the open state of the receptor and enhance ion flux. In this study, we assay integrated agonist-stimulated ion efflux over 15 msec, as a measure of receptor activity. In nicotinic acetylcholine receptor-rich membrane vesicles from *Torpedo*, we show that, in contradiction to this elegant model, long chain and short chain alkanols appear to act at different sites. Firstly, ethanol and octanol do not compete for a single site on the receptor. Secondly, alkanol chain length dependencies for inhibition and for flux enhancement are significantly different. Thirdly, intermediate length alkanols do not partially inhibit channels, as required by the model; high concentrations of these alkanols completely inhibit the response. Fourthly, careful measurements, including determination of the free alkanol concentration, of inhibitory potencies of alkanols from propanol to decanol show no evidence for a steric contribution to the ability of an alkanol to inhibit the ion channel. Furthermore, our results suggest that the inhibitory effect of long chain alkanols may be mediated by a discrete site on nicotinic acetylcholine receptors, whereas there is no evidence that a protein site is involved in the excitatory mechanism of short chain alkanols. Indeed, it seems more likely that short chain alkanols may have a nonspecific site of action.

The nAcChoR is the most abundant and best characterized of the synaptic ion channels (for review see Ref. 1) and has classically been used as a model system in which to study the effect of anesthetics on receptor activity. Direct effects of *n*-aliphatic alcohols (alkanols) have been characterized electrophysiologically at amphibian and mammalian NMJs (2–6) and using ion flux measurements at *Torpedo* nAcChoR (7, 8). The action of short chain alkanols differs qualitatively from that of long chain alkanols. Long chain alkanols, like other general anesthetics, inhibit cholinergic postsynaptic endplate currents in vertebrates, whereas alkanols shorter than pentanol appear to enhance nAcChoR-mediated cation flux.

Bradley *et al.* (9) proposed that the different actions of alkanols could be accounted for by interaction at a single hydrophobic site within the channel lumen; long chain alkanols are large enough to completely block the channel and so inhibit ion flux, whereas short chain alkanols are too small to block and, in binding to the channel, stabilize the open state to result in enhanced flux activity.

However, the electrophysiologically determined concentration-response curves used to support this model, and even the 10-sec flux assay used earlier by this laboratory, involve relatively long exposures of nAcChoR to alkanol and agonist solutions. Because all alkanols are known to enhance slow desensitization (7), these results are difficult to interpret simply. Any changes in receptor desensitization will underlie measured concentration-response curves and will distort apparent alkanol interactions. To avoid these and other problems (8), in this work we have measured the action of alkanols over a 15–20-msec time interval. Preliminary measurements of the rate of receptor desensitization in the presence of octanol have shown that flux kinetics remain linear for approximately 15–20 msec and, for octanol at least, onset of alkanol action occurs within the experimental time frame (10). Therefore, 15 msec was

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ABBREVIATIONS: nAcChoR, nicotinic acetylcholine receptor; NMJ, neuromuscular junction; AcCho, acetylcholine; CCho, carbachol; DFP, diisofluorophosphate; TPS, *Torpedo* physiological saline; SC_{50}, alkanol concentration which doubles agonist affinity; MEPC, miniature endplate current.

chosen as a suitable time over which to integrate ion flux, because it would exclude any effects of desensitization on the measured flux response and also would provide sufficient signal to obtain inhibitory dose-response curves for long chain alkanols.

The single site hypothesis of Bradley *et al.* (9), in which potency depends on both binding affinity and steric factors, predicts that the chain length dependency of alkanol inhibition should be nonlinear. Alkanols that are large enough to completely block the channel only depend on hydrophobic interactions for inhibition and potencies should parallel the membrane/buffer coefficient (11). The potencies of shorter partial inhibitors will depend on both size and hydrophobicity and so should have a steeper chain length relationship than the membrane/buffer partition coefficient. To obtain a more detailed and precise determination of the chain length dependencies of alkanol actions than has been available heretofore, we have measured the effects of all 1-alkanols, from methanol to decanol, on ion flux integrated over 15 msec, using gas chromatographic analysis to check the alkanol concentrations employed.

Materials and Methods

Preparation of *Torpedo* postsynaptic membranes. Postsynaptic membranes from freshly dissected electroplaques of *Torpedo nobiliana* (Biofish Associates, Georgetown, MA) were prepared using sucrose density gradient centrifugation at 4°, essentially as described by Braswell *et al.* (12). Membrane suspensions [5–10 mg of protein/ml by the method of Lowry *et al.* (13) and 7–15 μM in [^{3}H]AcCho binding sites, assayed as described (12)] were kept frozen at −80° for up to 6 months and thawed within 48 hr of use.

Measurement of nAcChoR cation channel function. Cation channel function was assayed by measurement of agonist-induced $^{86}Rb^+$ efflux from sealed native *Torpedo* electroplaque vesicles, using either a manual 10-sec ion flux measurement or a 5–30-msec quenched-flow technique, as described by Forman *et al.* (14). The number of active receptor-channel complexes was reduced by blocking with the irreversible inhibitor α-bungarotoxin, to prevent full equilibration of tracer within the experimental assay time (14). Channel activity was stimulated with either CCho, an AcCho analogue that is not hydrolyzed by acetylcholinesterase, or AcCho. When AcCho was used, acetylcholinesterase was inhibited by treatment of the vesicles with 0.1 mM DFP for 20 min before flux assays. DFP had no effect on flux response. Total agonist-stimulated $^{86}Rb^+$ counts ($cpm_{Ag,t}$) were corrected for passive, time-dependent $^{86}Rb^+$ leak from sealed vesicles ($cpm_{leak,t}$). The corrected efflux response is expressed as F_A, the percentage of non-leak $^{86}Rb^+$ counts released (14):

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

Any alkanol-induced enhancement of $^{86}Rb^+$ leak from sealed vesicles was detected as an increase in filtrate cpm values above the leak level without alkanol and was analyzed similarly to agonist-induced efflux (Eq. 1).

Reversibility of alkanol actions. The reversibility of alkanol effects after a 10-sec exposure in the absence of agonists was tested essentially as described (8). Briefly, vesicles were incubated with alkanol for 10 sec and then leak and CCho-induced flux measurements were made, either in the presence of the same concentration of alkanol or after 10-fold back-dilution in TPS (250 mM NaCl, 50 mM Na_2HPO_4, 5 mM KCl, 2 mM $MgCl_2$, 3 mM $CaCl_2$, 0.02% sodium azide). Controls were performed by the same method but with one tenth the alkanol concentration throughout.

Preparation of *n*-alkanol solutions. Solutions of methanol, ethanol, propanol, and butanol in TPS were prepared by weighing of the alkanols into TPS in partially filled volumetric flasks and adjustment with TPS to the final volume. Ice-cold TPS was used to prevent precipitation of calcium phosphate (8), and solutions were used immediately at 4°. For hexanol, octanol, and decanol, saturated stock solutions in TPS (61 mM, 4.0 mM, and 0.3 mM, respectively) were prepared by overnight stirring of excess alkanol in TPS at 4°. All alkanol concentrations were measured by gas chromatography immediately before use. For long chain alkanols, which adhered to the glass storage vessels to some extent, concentrations were measured both before and after passage through the rapid mixing device, in order to quantitate the exact alkanol concentration experienced by the nAcChoR-rich vesicles. No alkanol was lost during the brief exposure to the Teflon tubes of the rapid mixing device, enabling precise measurement of alkanol concentration-response curves.

Chemicals. DFP was from Aldrich Chemical Co. (Milwaukee, WI); α-bungarotoxin, buffer reagents, AcCho, CCho, and procaine hydrochloride were from Sigma Chemical Co. (St. Louis, MO). [^{3}H]AcCho and ^{86}RbCl were from New England Nuclear (Boston, MA). Anhydrous ethanol (≥99.9% purity) was from Pharmco (Dayton, NJ). Methanol (≥99.9% purity), propanol (≥99.8% purity), butanol (≥99.7% purity), hexanol, heptanol, octanol, nonanol, and decanol were from Aldrich or Sigma and were at least 99% pure, as determined by gas chromatography.

Results

Alkanol effects on passive $^{86}Rb^+$ leak. All the alkanols are known to disorder the *Torpedo* membrane, with the potency of this effect increasing regularly with chain length (7). Thus, the effect of alkanols on the passive leak of $^{86}Rb^+$ from sealed *Torpedo* vesicles was tested. Although the alkanols all increased the 10-sec leak from membrane vesicles, this was significant only at high concentrations. When necessary, the passive leak of $^{86}Rb^+$ from vesicles was measured simultaneously with each experimental point at the appropriate alkanol concentration, to correct for enhanced leak.

The relative potencies of alkanols to induce membrane leakiness were assessed by determination of concentration-response curves for passive $^{86}Rb^+$ leak over a 1-min time interval. Concentrations required to induce 50% of trapped $^{86}Rb^+$ to leak from the vesicles in 1 min decreased with chain length, ranging from 1300 mM for ethanol to 2.5 mM for octanol.

Alkanol concentration-response curves. Alkanol concentration-response curves (Fig. 1) were established by measurement of inhibitory effects on maximal AcCho-stimulated quenched-flow $^{86}Rb^+$ efflux ($F_{A_{max}}$ at 1 mM AcCho). Briefly, all alkanols except methanol and ethanol inhibited maximal stimulated ion flux. Concentration-inhibition data were analyzed by iterative nonlinear least squares fit to a logistic equation:

$$F_A^{alk} = F_{A_{max}} \times \left[1 - \frac{I^{n_I}}{(I^{n_I} + K_I^{n_I})}\right] \quad (2)$$

where I is the alkanol concentration, K_I is the 50% inhibiting concentration, and n_I is the Hill coefficient. Fitted parameters are summarized in Table 1.

We chose 15 msec as a suitable flux integration time, during which flux kinetics remained approximately linear and sufficient signal was obtained to accurately measure inhibitory dose-response curves. Alkanols from propanol to decanol showed complete flux inhibition, with their potencies increasing linearly with chain length. IC_{50} potency increased on average 25-fold with the addition of each methylene group, with a plot of $\log_{10}$ (IC_{50}) versus carbon number having a slope of −0.70 ±

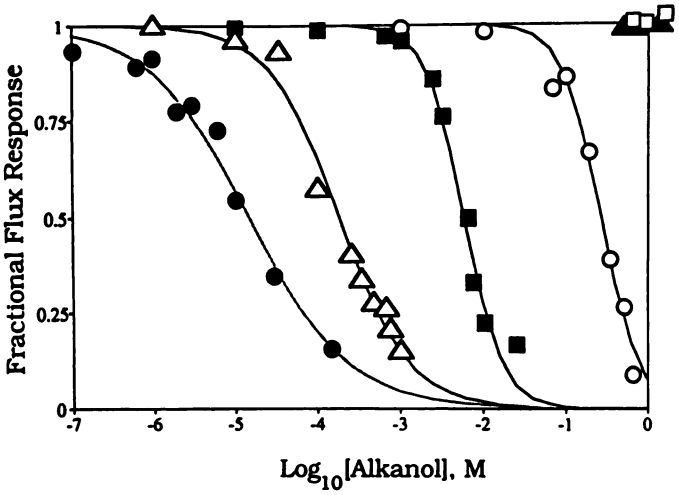

Fig. 1. Alkanol concentration-response curves measured using rapid quenched flow. The effects of alkanols on $^{86}Rb^+$ efflux responses to 1 mM AcCho were measured over 15 msec. Responses were normalized to $F_{A_{max}}$ measured under the same conditions without alkanols. Data were fitted to Eq. 2 by nonlinear least squares regression and are given in Table 1. □, Methanol; ▲, ethanol; ○, propanol; ■, pentanol; △, heptanol; ●, nonanol.

TABLE 1

Fitted parameters for alkanol inhibition and enhancement of AcCho-induced flux over 15 msec

The inhibitory and excitatory effects of alkanols on $^{86}Rb^+$ efflux responses to AcCho were measured over 15 msec. Responses were normalized to $F_{A_{max}}$ under the same conditions without alkanols. Inhibitory data were fitted to Eq. 2 by nonlinear least squares regression, as shown in Fig. 1, to obtain K_I values. Excitatory data were fitted to Eq. 3 by nonlinear least squares regression, as shown in Fig. 3, to obtain SC_{50} values.

Alkanol	K_I	n_I	SC_{50}	K or C[a]
	mM		mM	mM
Methanol			575	
Ethanol			270	
Propanol	270 ± 21	1.8 ± 0.25	61	373
Butanol	26 ± 2.6	1.9 ± 0.30	17[b]	35
Pentanol	6.0 ± 0.37	1.9 ± 0.23		4.8
	μM			μM
Hexanol	940 ± 67	1.5 ± 0.10		640
Heptanol	190 ± 25	1.0 ± 0.30		110
Octanol	43.6 ± 0.41	1.3 ± 0.30		55
Nonanol	11.3 ± 0.27	0.7 ± 0.10		
Decanol	2.6 ± 0.16	1.1 ± 0.36		

[a] $K = k_{-2}/k_2$; C = half-height concentration for alkanol channel blockade at mouse neuromuscular junction. From McLarnon *et al.* (6).
[b] 10-sec flux measurement.

0.021. Gibb's free energy for flux inhibition for each alkanol can be evaluated from $\Delta G^0 = -RT \cdot \ln K$ and it increased by 3.7 ± 0.11 kJ/CH_2 group. This is consistent with values reported by Morrell (3.3 kJ/CH_2)[1] and derived from data of McLarnon *et al.* (4.3 kJ/CH_2) (6). Concentrations of up to 3.0 M methanol or up to 2.0 M ethanol failed to show any effect on maximal AcCho-stimulated flux. Table 1 reveals that Hill coefficients (n_I) for the *n*-alkanols decreased from values near 2.0 for propanol and butanol to values near 1.0 for decanol.

These inhibitory effects of long chain alkanols in $^{86}Rb^+$ flux experiments are consistent with the observed decreases in channel lifetime at the NMJ (5, 15). Early observations of the action of octanol (15) were made with 0.1 to 1.0 mM, whereas octanol blockade of flux in *Torpedo* vesicles is characterized by a K_I of 50 μM at 15 msec. Others (5, 9) have observed significant reductions in ion flux at the NMJ in the presence of 50 to 100 μM octanol. K_I values for long chain alkanols derived from our 15-msec flux measurements are in excellent agreement with those reported by McLarnon *et al.* (6) using MEPC analysis at mouse endplates (see Table 1).

Alkanol effects on agonist concentration-response curves. Further differences between short and long chain alkanols were observed when effects on agonist concentration-response curves at all time points were tested. Leak-corrected flux responses were analyzed by fitting to another logistic equation:

$$F_A^{alk} = F_{A_{max}} \times \frac{[A]^{n_H}}{[A]^{n_H} + K_A^{n_H}} \quad (3)$$

where A is the agonist concentration, K_A is the 50% activating concentration, and n_H is the Hill coefficient.

Fig. 2 shows that, at 15 msec, 25 μM octanol reduced $F_{A_{max}}$ by 33% but had no effect at all on K_A. In contrast, propanol, ethanol, and methanol all caused leftward shifts in AcCho concentration-response curves. This resulted in an apparent increase in affinity for AcCho (characterized by a decreased fitted K_A for the agonist concentration-response curve) and enhanced $^{86}Rb^+$ efflux at low agonist concentrations. This leftward shift of agonist concentration-response curves has been observed using the 10-sec manual ion flux assay with ethanol and is identical to the effect seen in rapid quenched-flow experiments (8).

In the absence of methanol, 6 μM CCho released 0.75% of trapped counts over 10 sec. A plot of $\log_{10}(F_A)$ from 10-sec flux data in response to 6 μM CCho *versus* methanol concentration

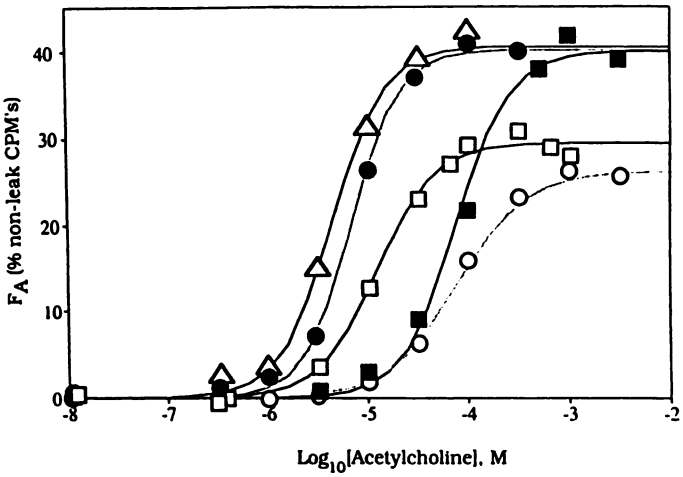

Fig. 2. Effect of *n*-alkanols on 15-msec AcCho concentration-response curves. $^{86}Rb^+$ efflux (15 msec) responses to AcCho were measured using rapid quenched flow both in the absence and in the presence of various alkanols. Alkanols were added simultaneously with AcCho during assays. Concentrations of methanol and ethanol were chosen arbitrarily, because these compounds do not inhibit $^{86}Rb^+$ flux. Concentrations of propanol and octanol were chosen to give approximately equipotent decreases in $F_{A_{max}}$, from the control without alkanol. AcCho concentration-response data were fitted by nonlinear least squares regression to Eq. 3. Fitted parameters for drawn lines are as follows: control (■), $F_{A_{max}} = 40$, $K_A = 77 \pm 2$ μM, $n_H = 1.60 \pm 0.30$; 3 M methanol (△), $F_{A_{max}} = 40.5 \pm 0.9$, $K_A = 4.5 \pm 0.3$ μM, $n_H = 1.63 \pm 0.17$; 1 M ethanol (●), $F_{A_{max}} = 40.1 \pm 0.8$, $K_A = 7 \pm 0.8$ μM, $n_H = 1.80 \pm 0.30$; 150 mM propanol (□), $F_{A_{max}} = 29.5 \pm 1.0$, $K_A = 12.5 \pm 1.4$ μM, $n_H = 1.48 \pm 0.27$; 25 μM octanol (○), $F_{A_{max}} = 26.2 \pm 0.7$, $K_A = 78.5 \pm 6.3$ μM, $n_H = 1.25 \pm 0.10$.

[1] R. Morrell, personal communication.

of up to 2.0 M produced a straight line, with a slope of 2.80 M^{-1} ($r = 0.93$), showing that F_A increased exponentially with methanol concentration. This result is consistent with the exponential dependence of 10-sec CCho-induced flux on ethanol concentration, which has a slope of 1.52 M^{-1} (9), and the exponential increase in channel lifetimes observed with short-chain alkanols at the vertebrate NMJ (3, 4).

Methanol causes only small changes in $F_{A_{max}}$ and, thus, it is possible to study its effects on K_A over very wide concentration ranges. Fig. 3 shows that the ratio of K_A in the presence of methanol (K_A^{alk}) to that in its absence (K_A^{con}) decreased exponentially as the methanol concentration increased (Fig. 3, *inset*). No saturation in effect was seen, and at 15 msec methanol did not appear to affect the Hill coefficient for the AcCho concentration-response curves. Similarly, Forman *et al.* (8) saw no identifiable saturation in the leftward shift induced by ethanol and no effect on the Hill coefficients for agonist concentration-response curves with up to 2 M ethanol.

We have previously defined a parameter, SC_{50}, as the alkanol concentration at which $K_A^{alk}/K_A^{con} = 0.5$ (8). SC_{50} values for methanol, ethanol, propanol, and butanol are reported in Table 1 and, again, decreased as chain length increased. The potencies of short chain alkanols for both enhancing $^{86}Rb^+$ flux (SC_{50} values) and slowing MEPC delay (3) paralleled the membrane/buffer or oil/water partition coefficient relationship (see Fig. 6 and Table 1).

No flux enhancement by submaximal concentrations of agonist was observed with alkanols longer than butanol.

Interactions between ethanol and octanol. In order to test whether short chain alkanols (represented by ethanol) interact at a common site with long chain alkanols (represented by octanol), additive effects of these two alkanols were assessed in both inhibitor and agonist concentration-response curves.

Fig. 4 shows that, using rapid quenched flow, 1.0 M ethanol had no effect at all on the inhibitory action of octanol over 15 msec of flux integration.

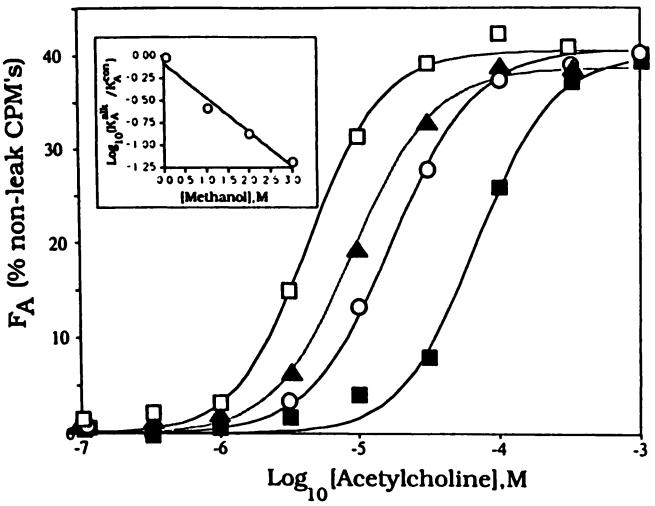

Fig. 3. Effect of methanol on 15-msec AcCho concentration-response curves. $^{86}Rb^+$ efflux (15 msec) responses to AcCho were measured using rapid quenched flow. Methanol, when present, was added simultaneously with AcCho during assays. AcCho concentration-response data were fitted by nonlinear least squares regression to Eq. 3. Fitted parameters for drawn lines are as follows: no methanol (■), $F_{A_{max}} = 40$, $K_A = 69.5 \pm 1.0$ μM, $n_H = 1.60 \pm 0.14$; 1 M methanol (○), $F_{A_{max}} = 40.3 \pm 0.9$, $K_A = 18.5 \pm 0.2$ μM, $n_H = 1.50 \pm 0.16$; 2 M methanol (▲), $F_{A_{max}} = 38.7 \pm 1.0$, $K_A = 9.4 \pm 0.1$ μM, $n_H = 1.50 \pm 0.21$; 3 M methanol (□), $F_{A_{max}} = 40.5 \pm 0.9$, $K_A = 4.5 \pm 0.3$ μM, $n_H = 1.63 \pm 0.25$.

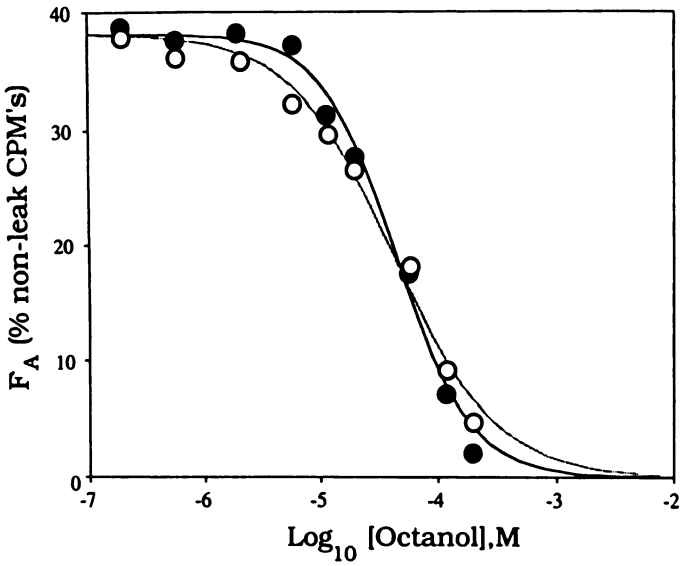

Fig. 4. Effect of ethanol on 15-msec octanol concentration-response curves. Octanol concentration-response data were measured at 15 msec, using rapid quench flow, in the presence and in the absence of 1.0 M ethanol. Alkanols were added simultaneously with 1 mM AcCho during $^{86}Rb^+$ efflux assays. Octanol concentration-response data were fitted by nonlinear least squares regression to Eq. 2. Fitted parameters for drawn lines are as follows: no ethanol (●), $F_{A_{max}} = 38 \pm 0.8\%$, $K_I = 45 \pm 4.3$ μM, $n_I = -1.4 \pm 0.15$; 1 M ethanol (○), $F_{A_{max}} = 38 \pm 2.2\%$, $K_I = 43 \pm 1.2$ μM, $n_I = -1.0 \pm 0.23$.

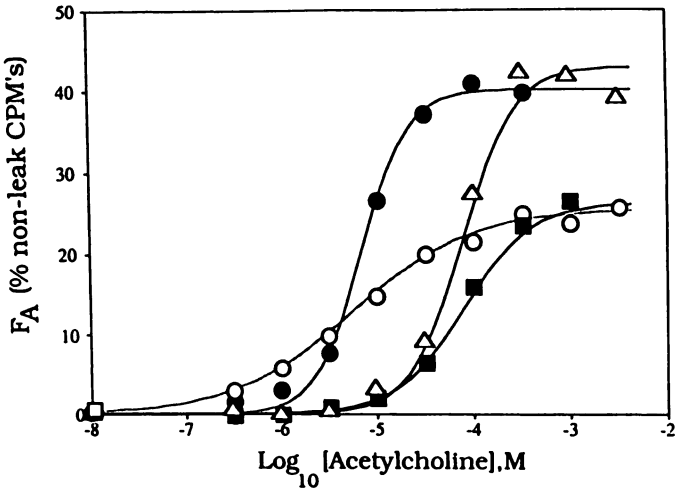

Fig. 5. Additive effects of octanol and ethanol on AcCho concentration-response curves measured using rapid quench flow. AcCho concentration-response data were measured at 15 msec using rapid quench flow. Alkanols were added simultaneously with 1 mM AcCho during $^{86}Rb^+$ efflux assays. AcCho concentration-response data were fitted by nonlinear least squares regression to Eq. 3. Alkanol conditions and fitted parameters for drawn lines are as follows: no alkanol (△), $F_{A_{max}} = 42.9 \pm 2.5$, $K_A = 77 \pm 2$ μM, $n_H = 1.6 \pm 0.3$; 1 M ethanol (●), $F_{A_{max}} = 40.1 \pm 0.8$, $K_A = 7 \pm 0.8$ μM, $n_H = 1.8 \pm 0.3$; 25 μM octanol (■), $F_{A_{max}} = 26.2 \pm 0.7$, $K_A = 78.5 \pm 6.3$ μM, $n_H = 1.3 \pm 0.1$; 25 μM octanol plus 1 M ethanol (○), $F_{A_{max}} = 25.5 \pm 0.9$, $K_A = 5.5 \pm 1$ μM, $n_H = 0.7 \pm 0.1$.

Fig. 5 shows the additive effects of ethanol and octanol on the full AcCho concentration-response curve. At 15 msec, 25 μM octanol reduced $F_{A_{max}}$ by about 30%, without affecting K_A, and 1.0 M ethanol had no effect on $F_{A_{max}}$, while reducing K_A

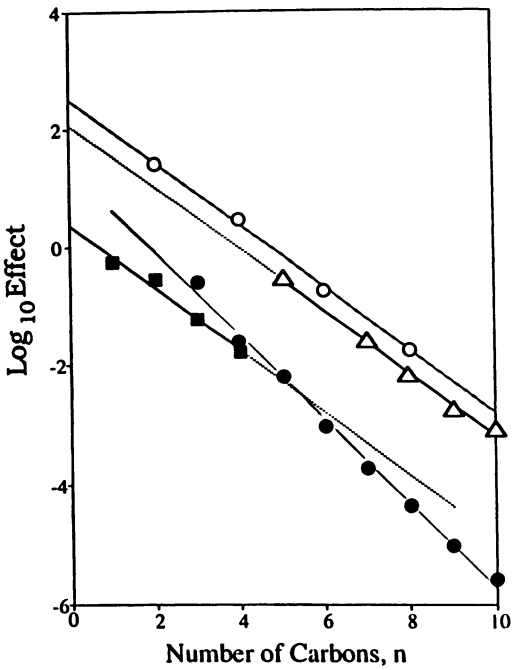

Fig. 6. Dependence on alkanol chain length of various effects on AcCho receptors. The change in membrane order parameter, $-\Delta S/M$ (○) [from Miller *et al.* (7)], 1/(erythrocyte membrane/buffer partition coefficient), λ (△) [from Seeman *et al.* (19)], 50% inhibition of maximal flux over 15 msec (●), and 2-fold leftward shift in AcCho concentration-response curves (■) are plotted against the number of carbon atoms in each alkanol (n). Errors are smaller than the symbols used. Slopes of linearly regressed data are as follows (all are given as $\log_{10}$ (effect)/CH_2 group): $-\Delta S$, −0.53 ± 0.016; partition coefficient, −0.53 ± 0.023; SC_{50}, −0.52 ± 0.046; K_I, −0.70 ± 0.021. Methanol (n = 1), SC_{50} = 575 mM; ethanol (n = 2), $-\Delta S/M$ = 0.038, SC_{50} = 270 mM; propanol (n = 3), K_I = 270, SC_{50} = 61 mM; butanol (n = 4), $-\Delta S/M$ = 0.351, K_I = 26 mM, SC_{50} = 17 mM; pentanol (n = 5), λ = 3.4, K_I = 6 mM; hexanol (n = 6), $-\Delta S/M$ = 5.49, K_I = 1 mM; heptanol (n = 7), λ = 39.0, K_I = 0.2 mM; octanol (n = 8), λ = 151.8, $-\Delta S/M$ = 51.8, K_I = 0.04 mM; nonanol (n = 9), λ = 582.1, K_I = 0.01 mM; decanol (n = 10), λ = 1226.3, K_I = 0.003 mM.

approximately 11-fold. A combination of both 25 μM octanol and 1.0 M ethanol demonstrated that ethanol did not significantly affect the blocking action of octanol and octanol exerted no effect on the excitatory action of ethanol. Previous studies, in which ion flux was assayed over 10-sec intervals, confirmed that octanol did not significantly change K_I^{app} in the presence of ethanol (7, 16). However, in the latter studies ethanol did appear to attenuate the inhibitory action of octanol slightly, but this effect was probably due to the enhancement by ethanol of fast desensitization during the 10-sec assay (8).

Reversibility of alkanol effects. To determine whether 10-sec exposures to 2.0 M methanol, 1.0 M ethanol, or 50 μM octanol caused irreversible functional effects at AcChoR, reversibility experiments were performed, as described in Materials and Methods. Octanol (50 μM) inhibited 80 ± 3% of the flux response to 5 mM CCho, and 10-fold back-dilution after 10 sec resulted in 3 ± 3% inhibition. In a control experiment, 5 μM octanol inhibited 5 ± 3% of $F_{5\,mM}$, indicating that the inhibitory actions of octanol were completely reversible during the 10-sec exposure.

Methanol (2.0 M) increased the flux response to 56 μM CCho from 15 ± 3% to 45.0 ± 2.0%. Ten-fold back-dilution after 10-sec exposure resulted in $F_{56\,\mu M}$ = 19.3 ± 0.8%, whereas the control experiment with 0.2 M methanol gave $F_{56\,\mu M}$ = 23.0 ± 1.4%. Again, this indicated complete reversibility of flux enhancement. Ethanol results were similar to those for methanol, as reported previously (8).

Discussion

Single-site model for alkanol action at nAcChoR. Bradley *et al.* (9) put forward a mechanism for alkanol action based on the sequential block model already proposed for local anesthetic action at nAcChoR (17, 18).

$$R_c \underset{a}{\overset{b}{\rightleftharpoons}} R_0 \underset{k_b}{\overset{k_f\cdot[\mathrm{Alk}]}{\rightleftharpoons}} R_B \qquad (4)$$

In this model all alkanols bind to a single hydrophobic site in the channel lumen, which is present only during the transient open conformation of the receptor (R_0). Long chain alkanols sterically occlude the ion channel and so convert the receptor to a blocked nonconductive state (R_B). In order for the channel to revert to the closed resting state (R), the alkanol must first dissociate from its binding site, returning the receptor to the open conformation. Short chain alkanols are too small to completely occlude the ion pore, and so the "blocked" state would still conduct ions to some extent. Thus, short chain alkanols would increase the apparent channel lifetime, which, at low agonist concentrations, would result in increased ion flux. At saturating agonist concentrations, where channels are open almost all of the time, partial block would cause a decreased flux response. The combination of these effects would produce a reduction in the maximum flux response and a leftward shift in agonist concentration-response curves.

However, this model cannot account for the observation that low concentrations of propanol and butanol produce flux enhancement and yet high concentrations also completely inhibit the flux response. No "partial" blocking of the channel is observed. Theoretically, according to the model, high concentrations of long chain alkanols should enhance ion flux at submaximal agonist concentrations, as do the shorter molecules, but the channel inhibition that must also occur at these concentrations would mask this effect. Estimations of SC_{50} for the long chain alkanols can be predicted from extrapolation of the data in Fig. 6. The high density of nAcChoR in unblocked *Torpedo* membrane preparations allows very high concentrations of an inhibitor to be used before the flux response is eliminated. No enhancement of flux at low agonist concentrations has been observed in our 15-msec experiments with up to 250 μM octanol, a concentration that should have a significant effect on agonist concentration-response curves (predicted SC_{50} = 150 ± 100 μM). Nor is any flux inhibition seen with methanol and ethanol, despite the fact that the concentrations studied, for ethanol at least, are well within the range required to produce significant inhibition, according to the model of Bradley *et al.* (9). The predicted K_I for ethanol from linear regression of the inhibition data in Fig. 6 is 0.8 ± 0.3 M, and yet no reduction in ion flux is seen even with as much as 3 M ethanol (8).

If the inhibitory action of alkanols is indeed due to simple steric occlusion of the open channel, then this should result in a biphasic dependence on chain length (see Ref. 11). The ability of short chain alkanols to inhibit the ion channel depends on both hydrophobicity and molecular size. Therefore, inhibitory potencies for short chain alkanols should have a steeper dependence on chain length than would some simple measure of

molecular hydrophobicity (e.g., membrane/buffer coefficient). Above some critical size, all alkanols are large enough to completely occlude the ion pore and inhibitory potency then depends solely on hydrophobicity. For alkanols longer than hexanol, the chain length dependency should flatten out and become parallel to the membrane/buffer partition coefficient dependency.

To test this prediction and obtain a detailed potency versus chain length plot for channel inhibition, we measured K_I values for the whole series of inhibitory alkanols, from propanol to decanol, at 15 msec. Because the shape and slope of this plot are so important, we analyzed all our alkanol solutions by gas chromatography to improve the accuracy of our measurements over those previously obtained (7).

As seen in Fig. 6, the potencies for channel inhibition can be fitted to a straight line. Membrane disordering, as reported by spin-labeled fatty acids, is the only measure of alkanol-membrane interactions that is available for *Torpedo* membranes at 4° (7), and these data are plotted in Fig. 6. The membrane/buffer partition coefficients for alkanols in another plasma membrane, the erythrocyte, are shown for comparison (19). Both membrane disordering and membrane partitioning show the same dependence on alkanol chain length.

Despite the addition of many new data points in this study, there is no indication that channel inhibition by long chain alkanols behaves as predicted by the model. Chain length dependency of inhibition by those alkanols large enough to block the channel completely, say from hexanol to decanol, does not parallel the plots for membrane disordering and partitioning. More importantly, inhibitory potencies do not show the biphasic chain length dependency discussed above; indeed, chain length dependency remains linear for the entire series of inhibitory alkanols.

However, as seen in Fig. 6, alkanol excitatory potencies have a linear chain length dependency that does parallel those measurements of membrane interactions shown. This suggests that, unlike channel inhibition, flux enhancement depends solely on some nonspecific membrane interaction.

Potencies for flux enhancement and flux inhibition are derived from the same experimental technique, are precisely obtained using gas chromatography analysis, and so are readily comparable. As shown in Fig. 6, the slopes of the logarithmic plots of K_I and SC_{50} versus chain length are clearly different ($p < 0.001$). Channel inhibition and flux enhancement appear to depend on different molecular properties for their potencies, which suggests that more than one site and mechanism are responsible for these actions. Additionally, dual-acting alkanols, namely propanol and butanol, bring about flux enhancement at much lower concentrations than those required for inhibition.

Our quenched-flow experiments showed no competitive interactions at all between short chain and long chain alkanols, which again suggests that they do not interact at the same site on the receptor. At 15 msec, ethanol did not affect octanol-induced flux inhibition and, likewise, octanol did not affect the apparent increase in agonist affinity induced by ethanol.

Thus, the different effects of alkanols at nAcChoR cannot be accounted for by actions at a single discrete site on nAcChoR, as suggested by Bradley *et al.* (9). It appears that at least two separate loci of action exist, and a multisite model for alkanol action must now be considered.

Alternative models for alkanol action at nAcChoR. It seems from our experiments that the inhibitory action of long chain alkanols is consistent with binding to a protein site, rather than being mediated through nonspecific membrane perturbation. Alkanols inhibit nAcChoR function at concentrations far below those that perceptibly perturb membranes, and Hill coefficients for channel inhibition tend to 1. However, the exact localization of the alkanol binding site and the mechanism of alkanol-induced inhibition can only be speculated upon.

Elegant studies by Changeux and colleagues (20–22) have localized the binding site of a local anesthetic, chlorpromazine, to a region of the nAcChoR, M2, thought to line the channel pore. Similarly, the site of affinity labeling has been reported for two other open channel blockers, all of which appear to be localized to the M2 region (23, 24). Single-channel studies reveal channel "flickering" in the presence of local anesthetics and alkanols, which is consistent with a rapid plugging of the open channel (6, 25). Thus, it is possible that long chain alkanols also inhibit nAcChoR by binding to, and sterically occluding, the open ionophore.

However, the local anesthetic procaine and octanol do not appear to act at the same site on nAcChoR (10), which suggests that, for these two compounds at least, there exist separate inhibitory sites on the receptor.

The alkanol inhibitory mechanism may not be as simple as the channel-plugging model for local anesthetics. Blocking and unblocking rates of alkanols decrease with temperature (6). Activation energies associated with channel blocking are in excess of 42 kJ/mol, higher than expected for simple diffusion, and channel blockade is associated with large increases in entropy. All this is consistent with some protein conformational change being associated with channel inhibition.

This suggests an alternative allosteric model of alkanol action, in which alkanols (ALK) bind to a site other than the channel lumen and induce a conformational change of the receptor to close the ion channel (6, 11).

$$O \rightleftharpoons O \cdot Alk \rightleftharpoons B \cdot Alk \qquad (5)$$

The ability of an alkanol to inhibit receptor activity depends on the $O \cdot Alk \rightleftharpoons B \cdot Alk$ equilibrium, where O and B are open and closed channels, respectively. Short chain alkanols such as methanol and ethanol cannot bring about this transition and so do not block the channel. All other alkanols can completely induce the conformational change required to close the channel; hence, complete flux inhibition is seen from propanol upwards.

Our alkanol concentration-response data show a downward trend in n_I values, from close to 2.0 for short chain alkanols to approximately 1.0 for heptanol and larger alkanols (Table 1). Franks and Lieb (26, 27) found that, for inhibition of firefly and bacterial luciferase by alkanols, a single hydrophobic inhibitory site of discrete molar volume existed on the protein, which could accommodate two molecules of short chain alkanols or one of a long chain alkanol. Whether this is the case for nAcChoR is not clear. For the longer chain alkanols, our results may well indicate that a single site on the nAcChoR mediates channel inhibition. However, it is equally possible that a number of sites, all with the same affinity, could produce the same observation. In any case, the analogy with the luciferases is not strong, because the dependence of luciferase inhibitory concentrations on chain length was far from linear.

Prior observation that slow desensitization, as measured by

ligand binding, is caused by the entire family of alkanols (7, 28, 29) suggests that desensitization and flux enhancement might both be produced by increases in the affinity of nAcChoR for agonists at sufficiently high concentrations of long chain alkanols. However, flux enhancement has been observed at times as fast as 5 msec, at which desensitization is negligible (8). This suggests that these two actions are independent.

The molecular mechanism of the enhancement of nAcChoR activity by ethanol has previously been discussed in detail (8). Our pharmacological study seeks not to elucidate the mechanism of action of short chain alkanols but to distinguish their site of action from that of the long chain alkanols. Our data do not strongly implicate either lipids or the AcChoR protein as the primary target site for short chain alkanols. Potencies for flux enhancement increase with alkanol chain length in parallel with membrane perturbations, suggesting that the increase in apparent agonist affinity is associated with some hydrophobic site. Indeed, because methanol and ethanol decrease K_I^{app} without saturation, there is no evidence that the action is due to binding to a saturable protein site.

Gage *et al.* (3) suggested that the increase in MEPC amplitude in the presence of ethanol was a separate effect from the inhibitory action of long chain alkanols and could be due to a change in the membrane dielectric constant. However, as discussed extensively by Forman *et al.* (8), flux enhancement by ethanol can be observed in the absence of a membrane voltage and so is unlikely to be due to changes in the electrical properties of the membrane bilayer. In our experiments, the SC_{50} values for short chain alkanols are in the concentration range where perturbations in membrane structure are detected using ESR probes (7, 30, 31). The parallel relationship between SC_{50} and membrane disordering (Fig. 6) suggests that bulk membrane lipids or lipids adjacent to AcChoR are indeed plausible targets for primary perturbations.

Conclusions. Thus, at nAcChoR there appear to be at least two alkanol-sensitive sites, which differ in their chain length requirements and their effect on receptor function. The different slopes of the K_I and SC_{50} versus chain length curves suggest the two groups of alkanols act at different sites. Alkanols that cause both flux enhancement and inhibition (propanol, butanol, and possibly pentanol) probably display these actions because they act at the different sites over a similar concentration range. It seems that very small alkanols, such as ethanol and methanol, do not bind to the channel-blocking site, and even high concentrations of octanol do not produce any flux enhancement.

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