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Cycloalkanemethanols Discriminate between Volume- and Length-Dependent Loss of Activity of Alkanols at the *Torpedo* Nicotinic Acetylcholine Receptor

SUSAN C.WOOD, W. ADAM HILL, and KEITH W. MILLER

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

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SUMMARY

Primary normal alcohols (1-*n*-alkanols) exert two effects on the nicotinic acetylcholine receptor when added simultaneously with agonist. First, propanol through decanol inhibit the open channel. Second, methanol through butanol, but not higher homologs, increase the apparent affinity of the agonist for inducing cation flux. To test the hypothesis that the length or volume of the alcohols might account for the fact that some members of the 1-*n*-alkanol homologous series lack activity, we have studied in parallel 11 members of another homologous series, i.e., the cycloalkanemethanols, c($C_nH_{(2n-1)}$)CH_2OH. With steadily increasing potency, agents from cyclopropanemethanol to cyclodecanemethanol completely inhibited carbachol-stimulated $^{86}Rb^+$ efflux from nicotinic acetylcholine receptor-rich postsynaptic vesicles from the electroplaques of *Torpedo nobiliana*, but even 90% saturated solutions of cycloundecanemethanol inhibited only part of the flux and neither cyclododecanemethanol nor cyclotetradecanemethanol caused any inhibition. Comparison of these results with those previously obtained for 1-*n*-alkanols indicates that as both series are ascended the cut-off in the inhibitory action on the channel occurs when the volume of the compounds exceeds approximately 340 Å^3. The apparent affinity for carbachol-induced flux was enhanced only by cyclopropanemethanol through cyclooctanemethanol, consistent with the hypothesis that a critical length of approximately 6.3 Å cannot be exceeded. Thus, the sites mediating the two effects have different steric requirements and may be physically distinct.

There is a consensus that general anesthetic agents act by disrupting neuronal conduction mechanisms, in particular by depressing transmission at excitatory synapses (1). However, despite extensive study, our knowledge of the actions of general anesthetics at the molecular level is still rudimentary. One approach has been to examine the effects of a homologous series of anesthetics on a well defined model system. In this way, the effects of systematic variation of one structural motif on drug potency and drug actions can be investigated, with a view to understanding the underlying molecular mechanisms. In most such series potency increases regularly with molecular weight, providing some information on the strength of drug-active site interactions. However, as molecular weight is further increased, at some point activity is suddenly lost (the cut-off point; reviewed in Ref. 2). This cut-off point often provides additional information about the nature of the site of action (3).

The best studied ligand-gated ion channel used in anesthesia studies is the nAcChoR (4–8). The extensive biochemical and functional characterization of this protein has been facilitated by the abundance of the receptor in the electric organs of certain species of fish (*Electrophorus* and *Torpedo*) and the availability of high affinity ligands, enabling a high yield of purified receptor protein to be obtained. This synaptic mediator of neuronal excitatory impulses has an amino acid sequence that is highly conserved between species (9, 10) and shows great homology with a variety of other ligand-gated ion channels.

General anesthetic agents have a number of effects on the nAcChoR (11). Of particular relevance to this study is that most, but not all, general anesthetics inhibit ion flux through the channel, and a number of the smaller ones enhance the apparent affinity of the agonist for channel opening. The homologous series of primary normal alkanols (1-*n*-alkanols) exhibit these effects at the nAcChoR. Alkanols from propanol to decanol inhibit tracer ion efflux from nAcChoR-rich vesicles when it is elicited by saturating concentrations of agonist (12–14). Short-chain alkanols with less than five carbon atoms enhance nAcChoR-mediated cation efflux when it is elicited by subsaturating concentrations of agonist, effectively increasing

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ABBREVIATIONS: nAcChoR, nicotinic acetylcholine receptor; IC_{50}, concentration causing 50% inhibition of agonist-stimulated flux; SC_{50}, concentration causing 50% reduction in carbachol concentration required to elicit 50% of maximum flux; TPS, *Torpedo* physiological saline.

the apparent affinity of the agonist for inducing the flux. However, it should be noted that methanol, ethanol, and dodecanol do not inhibit agonist-induced ion flux, and pentanol and higher 1-*n*-alkanols fail to increase the apparent affinity of the agonist for channel opening. Although recent studies suggest that these two effects of 1-*n*-alkanols are independent, the molecular mechanisms of each action have not been resolved (14). Part of the problem is that it is impossible to conclusively promote any one molecular model of drug action based on the study of a single homologous series of compounds, because incremental changes in a structural motif vary not only the parameter of interest but also many other physicochemical properties, any of which could be correlated with potency. This ambiguity can be resolved by considering data from more than one homologous series of drugs, because then physical properties will vary independently for each structural motif.

For this reason, we have recently introduced a new series of alkanols, the cycloalkanemethanols, $c(C_nH_{(2n-1)})CH_2OH$, to studies of general anesthesia (15). In this series length increases less rapidly with the number of methylene groups than it does in the 1-*n*-alkanol series, whereas volume increases at about the same rate. *In vivo*, the actions of these agents resembled those of the 1-*n*-alkanols, in that their general anesthetic potencies increased with the number of methylene groups and anesthetic activity was lost beyond cyclododecanemethanol, compared with dodecanol in the 1-*n*-alkanol series. These findings indicate that the volume of an alcohol is a better predictor of the general anesthetic cut-off point than is its length. The present study extends this approach to an *in vitro* preparation, the nAcChoR-rich synaptic membranes from *Torpedo nobiliana*.

Experimental Procedures

Materials. *T. nobiliana* were purchased from Biofish Associates (Georgetown, MA). Cyclic ketones were purchased from Aldrich Chemical Co. (Milwaukee, WI). Cycloalkanemethanols were purchased either from Aldrich Chemical Co. (Milwaukee, WI) or from Columbia Organic Chemicals (Cassatt, SC) or were synthesized (see below). All cycloalkanemethanols were >99% pure, as determined by gas chromatography. $^{86}Rb^+$ was supplied by New England Nuclear (Boston, MA) with an activity of 5 mCi/ml. Carbamylcholine and α-bungarotoxin were purchased from Sigma Chemical Co. (St Louis, MO).

Synthesis of cycloalkanemethanols. Cyclononanemethanol, cyclodecanemethanol, and cyclotetradecanemethanol were synthesized by ring contraction of the appropriate cyclic ketone according to the method of Vincek *et al.* (16). The structure and purity of the compounds were confirmed by NMR and gas chromatography. All compounds were >99% pure.

Purification of cycloundecanemethanol. Cycloundecanemethanol was supplied by Aldrich at a purity of 80%. It was purified by column chromatography using a silica column eluted with toluene. The cycloundecanemethanol recovered was >99% pure, as determined by gas chromatography.

Preparation of membranes. Synaptic membrane vesicles rich in nAcChoR were prepared from freshly dissected *T. nobiliana* electroplaque by differential and sucrose density gradient centrifugation, as described previously (17). The resultant membranes were divided into 1-ml aliquots, frozen in liquid nitrogen, and stored at −80°. These aliquots were thawed as needed, stored under nitrogen at 4°, and used within 3 days. Specific activity of the membrane preparations and results were unaffected by storage conditions.

Measurement of $^{86}Rb^+$ efflux from vesicles. Flux assays were performed at 4° in TPS, using the methods described previously (8, 18). Synaptic vesicles were incubated overnight with $^{86}Rb^+$ and enough α-bungarotoxin to block 20% of the maximum $^{86}Rb^+$ efflux elicited by 5 mM carbachol, to prevent the vesicles emptying of $^{86}Rb^+$ before termination of the assay, as explained in Ref. 8. The vesicles were separated from the excess $^{86}Rb^+$ using a Sephadex G-50 chromatography column. After 20 min the vesicles were passed down a second column (Dowex 50W, 20–50 mesh) to remove passively leaking $^{86}Rb^+$. The vesicles (100 μl) were then added to the test solution (900 μl) containing either carbachol or carbachol plus alcohol, vortex mixed, and after 10 sec vacuum filtered through Whatman GF/F filters. Aliquots (500 μl) of the filtrate were then added to a scintillation cocktail (Poly-fluor; Packard) and counted with a Tri-carb 1900CA scintillation counter. 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 fraction of non-leak $^{86}Rb^+$ counts released:

$$F_a = \frac{[\text{cpm(Ag},t) - \text{cpm(leak},t)]}{[\text{cpm(total)} - \text{cpm(leak)}]} \quad (1)$$

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.

Data analysis of flux experiments. IC_{50} values were calculated by fitting the data with an iterative nonlinear least squares method to a logistic equation:

$$\frac{F_a^{alk}}{F_a(\text{max})} = \left(1 - \frac{I^n}{I^n + IC_{50}{}^n}\right) \quad (2)$$

where flux in the presence of a saturating agonist concentration is denoted by $F_{a(max)}$ in the absence and F_a^{alk} in the presence of alkanols, I is the alkanol concentration, IC_{50} is the concentration of alkanol inhibiting 50% of the maximal agonist-induced ion flux, and n is the Hill coefficient.

Agonist concentration-response curves were analyzed by fitting to another logistic equation:

$$\frac{F_a^{alk}}{F_a(\text{max})} = \frac{A^n}{A^n + K_a{}^n} \quad (3)$$

where F_a is flux elicited by various concentrations of agonist and $F_{a(max)}$ is that obtained at saturating agonist concentrations, A is the agonist concentration, K_a is the concentration of agonist that elicits 50% of the maximal ion flux, and n is the Hill coefficient.

Aqueous solubilities. Saturated solubilities of cycloalkanemethanols in TPS at 4° were determined by gas chromatography, using a Hewlett Packard 5890 gas chromatograph and a flame ionization detector. Excess cycloalkanemethanol was added to a 5-ml vial containing 3 ml of TPS and a small glass tube that penetrated the solution. The vial was sealed and gently stirred overnight at 4° to equilibrate. Samples of 1 μl were extracted from the aqueous phase through the glass tube, to avoid contact with the undissolved alcohol. The sample was injected onto a J & W Megabore DB5 glass capillary column (J & W, Folsom, CA), at temperatures between 35° and 160°. The integrals of the peak areas of at least four samples of each saturated solution of alcohol were averaged and the saturated concentration was determined from an average of at least four peak area values from at least two different standard solutions. Standards were made gravimetrically and generally encompassed a range between 10 and 80% of the saturated solubility. Standards were analyzed concurrently with the saturated solutions. The flame ionization detector gave a linear response over the alcohol concentration range examined (1.8 M to 15 μM).

Partition coefficients. Partition coefficients for the cycloalkanemethanols were determined by partitioning the alcohols between oleoyl alcohol and water at 22°. The separate layers were analyzed by gas chromatography. The integrals of the peak areas of at least three samples of each phase were averaged and the partition coefficients

were determined from an average of at least seven peak area values from the different phases. Oleoyl alcohol/water partition coefficients have been used in previous anesthesia studies and were used herein in preference to octanol/water partition coefficients because of the difficulty in separating the cycloalkanemethanols from octanol by gas chromatography.

Molecular modeling. Length of the alcohols was determined using Alchemy II molecular modeling software (Tripos Associates, St. Louis, MO). Structures were either produced from X-ray crystallography data or drawn freehand; in either case the structure was extended to its maximum length and then energy minimized using an MM2-based calculation, and the distance was measured between the hydroxyl oxygen and the farthest carbon atom.

Molecular volume was determined by dividing the molar volume (molecular weight/density) by Avogadro's number, with the result being expressed in cubic angstroms. Densities were provided by the manufacturer. The molecular volumes of the solid cycloalkanemethanols were calculated by extrapolation of the values for the liquid cycloalkanemethanols.

Results

Physicochemical Properties

The physicochemical properties of the cycloalkanemethanols determined in this study are shown in Table 1. Overall, the length, as measured from the hydroxyl oxygen to the farthest carbon in the ring, increases in an incremental manner with increasing number of methylene units. However, there is a slight deviation from a linear relationship due to the asymmetry of the alkane ring; rings with an odd number of carbons have two carbons equidistant from the hydroxyl oxygen, whereas those containing an even number of carbons have only one. This periodicity results in a relatively larger increase in length per carbon unit on moving from an even-numbered alkane ring to an odd-numbered one and is most significant with the smallest cycloalkanemethanols. On the other hand, the molecular volume increases linearly with an almost constant increment of 24 Å^3/methylene unit added to the ring.

The saturated solubilities of the cycloalkanemethanols were determined in TPS at 4°. Cyclopropanemethanol was fully miscible with water. The remaining cycloalkanemethanols had saturated solubilities that decreased exponentially with increasing number of methylene groups in the ring, ranging from 1.8 M for cyclobutanemethanol to 37 μM for cyclododecanemethanol (Table 1).

The oleoyl alcohol/water partition coefficients of the cycloalkanemethanols increased approximately 3-fold with each additional methylene group, ranging from 0.38 for cyclopropanemethanol to 60 for cyclooctanemethanol (Table 1). Cycloalkanemethanols larger than cyclooctanemethanol could not be reliably separated from the oleoyl alcohol on the gas chromatograph.

TABLE 1

The physicochemical properties of the cycloalkanemethanols

The parameters were determined as described in the text. ∞ indicates infinite solubility. ND indicates not determined.

Name	Maximum length	Molecular volume	Saturated solubility	Partition coefficient (oleoyl alcohol/water)
	Å	Å^3	mM	
Cyclopropanemethanol	3.86	134	∞	0.38
Cyclobutanemethanol	4.29	156	1800	1.1
Cyclopentanemethanol	4.86	180	470	5.6
Cyclohexanemethanol	5.24	204	86	15
Cycloheptanemethanol	5.79	228	7.2	48
Cyclooctanemethanol	6.28	250	6.3	60
Cyclononanemethanol	6.74	273	1.95	ND
Cyclodecanemethanol	7.20	296	0.55	ND
Cycloundecanemethanol	7.70	320	0.21	ND
Cyclododecanemethanol	8.20	340	0.037	ND
Cyclotetradecanemethanol	8.52	388	ND	ND

Pharmacological Properties

Cycloalkanemethanol flux-inhibition curves. Cycloalkanemethanol flux-inhibition curves were established by measuring inhibitory effects on maximal carbachol-stimulated 10-sec $^{86}Rb^+$ efflux and fitting the experimental data to eq. 2. All of the cycloalkanemethanols tested, with the exceptions of cyclododecanemethanol and cyclotetradecanemethanol, inhibited maximally stimulated ion flux in a concentration-dependent manner, with Hill coefficients mostly close to 1 (Fig. 1). Fitted parameters for all cycloalkanemethanols are summarized in Table 2. Inhibitory potencies for the cycloalkanemethanols up to cycloundecanemethanol increased exponentially with increasing number of methylene units, and the compounds produced complete inhibition of maximally stimulated ion flux

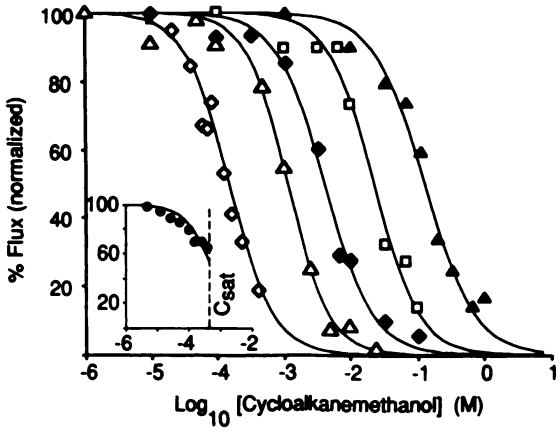

Fig. 1. Inhibition curves for carbachol (5 mM)-stimulated $^{86}Rb^+$ efflux for a series of cycloalkanemethanols. ▲, Cyclopropanemethanol; □, cyclobutanemethanol; ♦, cyclopentanemethanol; △, cyclohexanemethanol; ◇, cyclooctanemethanol; ●, cycloundecanemethanol. The *curves* were drawn using eq. 1, with percentage flux (normalized) = 100 × [F_e^{alk}/$F_{e(max)}$] and the parameters given in Table 2. *Inset*, inhibition curve for cycloundecanemethanol. *Vertical dashed line labeled* C_{sat}, highest achievable concentration of the agent in the buffer.

TABLE 2

Inhibition of carbachol-induced cation flux

Analysis of the flux inhibition curves using nonlinear least squares fitting to Eq. 1 yielded the concentration of cycloalkanemethanol required to inhibit 50% of the maximum flux (IC_{50}) and the Hill coefficient (n_H). Values shown are means ± standard deviations.

Alcohol	IC_{50}	Hill coefficient
	mM	
Cyclopropanemethanol	125 ± 9.0	1.15 ± 0.11
Cyclobutanemethanol	22.3 ± 0.24	1.3 ± 0.14
Cyclopentanemethanol	4.1 ± 0.32	1.2 ± 0.11
Cyclohexanemethanol	1.13 ± 0.09	1.4 ± 0.16
Cycloheptanemethanol	0.30 ± 0.091	1.67 ± 0.09
Cyclooctanemethanol	0.15 ± 0.087	1.3 ± 0.12
Cyclononanemethanol	0.034 ± 0.0029	1.4 ± 0.16
Cyclodecanemethanol	0.015 ± 0.0056	1.1 ± 0.06
Cycloundecanemethanol	1.04 ± 0.095	0.85 ± 0.08
Cyclododecanemethanol	I*	
Cyclotetradecanemethanol	I	

* I indicates ineffective at concentrations up to 90% of a saturated solution.

well before they reached their saturated solubility limits. The IC_{50} values ranged over nearly 4 orders of magnitude upon addition of more methylene groups, with the inhibitory potency increasing >8000-fold between cyclopropanemethanol and cyclodecanemethanol. However, cycloundecanemethanol only partly inhibited ion flux; its inhibition curve intersects the limit of its saturated solubility, such that even a 90% saturated solution of this compound produces only about 40% inhibition (see Fig. 1, *inset*). It is possible to extrapolate the experimental data to obtain a theoretical value for the IC_{50} of cycloundecanemethanol, and this is given in Table 2. This value is comparable to that of cyclohexanemethanol and is approximately 1000 times less potent than would be expected from the relationship between inhibitory potency and carbon number established with the smaller cycloalkanemethanols.

Cycloalkanemethanols larger than cycloundecanemethanol were completely devoid of inhibitory activity even when used at concentrations close (~90%) to their saturated solubility limit. In these cases inhibitory activity was not tested after prolonged preincubation of the compound, both to be consistent throughout the series of tests and to avoid anesthetic-induced desensitization (11, 19).

Cycloalkanemethanol effects on carbachol concentration-response curves. Carbachol concentration-response curves were measured for 1 μM to 5 mM carbachol in the presence and absence of various fixed concentrations of the cycloalkanemethanols. Because all cycloalkanemethanols (up to cycloundecanemethanol) inhibited the flux response, the amount of α-bungarotoxin used to block the receptor preparation was adjusted in each experiment to ensure that sufficient signal remained for accurate measurement of the carbachol concentration-response curve. Leak-corrected flux responses were analyzed by fitting to eq. 3.

The control carbachol concentration-response curve was sigmoidal in shape, with a plateau between 0.5 and 5 mM. The curve had a K_a of 87 ± 4.1 μM and a Hill coefficient of 2.0 ± 0.18, in good agreement with previous work (11, 13, 18). When the carbachol concentration-response relationship was determined in the presence of various fixed concentrations of cycloalkanemethanol, all of the cycloalkanemethanols up to cyclooctanemethanol caused the measured concentration-response curves to be shifted to lower concentrations, without alteration in shape, in a manner similar to that reported previously for the 1-*n*-alkanols (11, 14). The values of K_a, obtained from a nonlinear least squares fit of the experimental data to eq. 3, decreased in the presence of each cycloalkanemethanol in a concentration-dependent manner.

Fig. 2 shows the relative change in K_a, expressed as $\log_{10}(K_a^{alc}/K_a^{con})$, as a function of the concentration of representative cycloalkanemethanols. Increasing concentrations of cycloalkanemethanol increase the apparent affinity for channel opening in a linear fashion, shifting the measured K_a to smaller values without showing any tendency toward saturation even at the highest concentrations studied (Fig. 2, *inset*). The potencies of the cycloalkanemethanols to elicit this effect increase linearly with the addition of methylene units, such that the magnitudes of the slopes of these relationships increase with increasing carbon number. This "flux-enhancing" effect has been reported previously for the 1-*n*-alkanols by Forman *et al.* (13), who quantified its magnitude by defining a parameter, SC_{50}, as the alcohol concentration at which K_a^{alc}/K_a^{con} is 0.5.

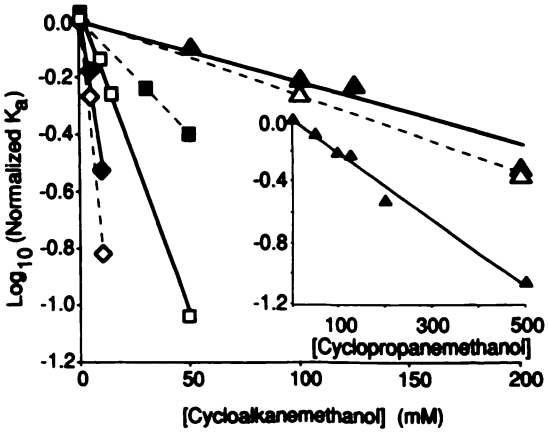

Fig. 2. Dependency of the apparent carbachol dissociation constant (K_a) on the concentration of cycloalkanemethanol. For each alcohol concentration the K_a values derived from fitting carbachol concentration-response curves to eq. 2 have been divided by the control value. The logarithm of this normalized K_a value is a linear function of alcohol concentration. The symbols indicate the same cycloalkanemethanols as in Fig. 1. ■, Cycloheptanemethanol. The concentrations of cyclohexanemethanol, cycloheptanemethanol, and cyclooctanemethanol have been multiplied by 100, to display all the data on one graph. The *line* of best fit was in each case determined by linear regression through the origin. *Inset*, the cyclopropanemethanol data are linear up to 500 mM, the maximum concentration examined.

TABLE 3

Ability of cycloalkanemethanols to shift the carbachol concentration-response curve to lower concentration

The slope is derived from linear least squares fitting through the origin of plots of the logarithm of the normalized carbachol K_a vs. cycloalkanemethanol concentration as shown in Fig. 2. The SC_{50} is the cycloalkanemethanol concentration that halves K_a (see text). Values shown are means ± standard deviations.

Alcohol	−Slope	SC_{50}	n^a
		mM	
Cyclopropanemethanol	0.00217 ± 0.00007	140 ± 17	9
Cyclobutanemethanol	0.0205 ± 0.0005	14 ± 1.4	6
Cyclopentanemethanol	0.050 ± 0.0023	5.8 ± 0.59	4
Cyclohexanemethanol	0.268 ± 0.001	1.12 ± 0.01	4
Cycloheptanemethanol	0.793 ± 0.001	0.380 ± 0.001	4
Cyclooctanemethanol	7.7 ± 0.38	0.037 ± 0.0059	4
Cyclononanemethanol	I[b]		2
Cyclotetradecanemethanol	I		2

[a] *n* is the number of carbachol concentration-response curves measured.
[b] I indicates ineffective at concentrations up to half-saturation (Table 1).

This parameter was derived for the cycloalkanemethanols from linear least squares fits of our data, normalized as in Fig. 2, and the values are given in Table 3. Their potencies increased regularly from cyclopropanemethanol to cyclooctanemethanol, but the larger members of the series tested did not produce a decrease in the measured values of K_a for carbachol-mediated ion flux concentration-response curves.

Discussion

Previous studies with 1-*n*-alkanols have characterized two actions of these compounds at the nAcChoR, i.e., an inhibitory action and a flux-enhancing effect (5, 12–14). The potencies for alcohol action at the nAcChoR can be correlated with a variety of molecular parameters, and a number of models have been proposed to account for these actions. These hypotheses can be divided into those proposing that all alcohols act at just one type of site in a size-dependent manner and those that

suggest that the different actions of large and small alcohols are mediated by separate mechanisms (5, 12, 20). Recent work has tended to suggest that the inhibitory and flux-enhancing actions are independent (14). This conclusion was based on a study of 1-*n*-alkanols in which it was observed that the chain length dependencies of the two actions were significantly different and that ethanol and octanol failed to perturb each other's actions. To determine whether these two hypothetical sites differ in their steric properties, we have studied the cycloalkanemethanol series.

Cycloalkanemethanols as an Alternative Series of Model Compounds

The family of cycloalkanemethanols provides a homologous series that complements the 1-*n*-alkanols. The alkane ring restricts the number of possible conformations that may be adopted, compared with the more flexible 1-*n*-alkanols, yet the hydroxyl group in both series remains unconstrained and can assume many spatial orientations, which is an advantage over the cycloalcohols (21) for our purposes.

This study examined the actions of the cycloalkanemethanols at the nAcChoR *in vitro*. Here we compare these data with previously published work on 1-*n*-alkanols from this laboratory (11, 13). We have chosen in this initial survey of the pharmacological properties of the cycloalkanemethanols to use the efficient flux assay, employing an integration time of 10 sec, rather than the the much more time-consuming flux efflux assay with millisecond time resolution. The advantage of the former method for the present purpose is that it minimizes problems caused by absorption of hydrophobic inhibitors, which can be quite serious in the quenched flux apparatus with its narrow tubes and consequent high surface area. On the other hand, this choice means that we are working in a time frame of nonlinear kinetics. Fortunately, however, our previous experience with the 1-*n*-alkanols shows that this does not influence the pharmacology. For the 1-*n*-alkanols, the relative shift in the apparent affinity of the agonist for stimulating flux (K_a) is independent of the flux integration period (13), and the IC_{50} values measured over 10 sec and within milliseconds are comparable (11). For example, for the five 1-*n*-alkanols between propanol and decanol for which we have data on both time scales, the ratio of IC_{50} values (millisecond/second) does not differ significantly from 1 ($p = 0.2$). This agreement probably arises because most of the measured flux occurs in milliseconds, the rate of fast desensitization is relatively insensitive to anesthetics, and slow desensitization takes minutes to develop (13). Thus, the quantitative flux data for 1-*n*-alkanols and cycloalkanemethanols can be directly compared and conclusions about structure-activity relationships safely drawn.

The cycloalkanemethanols behave qualitatively in much the same way as do the 1-*n*-alkanols (11, 14). First, both series inhibit ion flux with potencies that increase with molecular weight up to a point at which the addition of another methylene group completely abolishes the inhibitory activity of the compounds. The cut-off effect occurs between decanol and dodecanol for 1-*n*-alkanols and between cycloundecanemethanol and cyclododecanemethanol for cycloalkanemethanols. No other data for brief exposures to long-chain 1-alcohols have been published. For more prolonged exposures desensitization makes interpretation more difficult,[1] but in electrophysiological studies of rat nAcChoRs the cut-off point for the 1-*n*-alkanol series was close to our estimate (7). Second, the lower molecular weight members of each series produce an apparent increase in the affinity for channel activation; 1-*n*-alkanols with up to four carbons and cycloalkanemethanols with up to nine carbons exert this effect. The two series differ only in that methanol and ethanol are unable to inhibit the nAcChoR, whereas even the smallest cycloalkanemethanols can do so.

Molecular Correlates of Cut-off in Alkanol Activity

Comparing the data for 1-*n*-alkanols and cycloalkanemethanols, it appears that the cut-off for inhibition of ion flux depends on the volume and not the length of the molecule. Cycloundecanemethanol, which has a length of 7.7 Å, is the largest cycloalkanemethanol to inhibit ion flux, whereas 1-*n*-alkanols as long as 12.5 Å (decanol) still inhibit flux (14). However, as shown in Fig. 3, the cut-off for inhibition of ion flux in the cycloalkanemethanol series occurs at a volume of <340 Å^3 (cyclododecanemethanol) but >320 Å^3 (cycloundecanemethanol). The anomalously low potency of cycloundecanemethanol might indicate that this molecule is on the borderline for the maximal volume permissible before potency is lost. The 1-*n*-alkanols exhibit cut-off between decanol (317 Å^3) and dodecanol (372 Å^3). Taken together, the data are consistent with a single inhibitory site that can bind alkanols with volumes of up to ~340 Å^3 (Fig. 3).

The two smallest members of the 1-*n*-alkanol series, methanol and ethanol, failed to inhibit ion flux at any attainable concentration (2, 12–14), whereas none of the cycloalkanemethanols failed to do so (Fig. 3). Ascending the series, the first 1-*n*-alkanol that has an inhibitory action (propanol) has length and volume (3.8 Å and 124 Å^3, respectively) similar to those of the smallest cycloalkanemethanol, cyclopropanemethanol (3.9

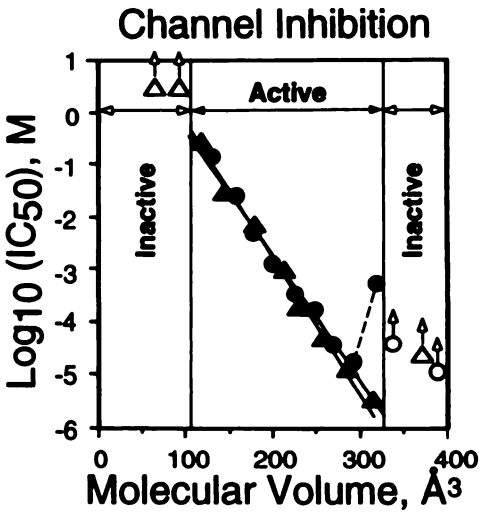

Fig. 3. The cut-off in the ability of alcohols to inhibit the ion channel of the nAcChoR correlates with their volume. The logarithm of the IC_{50} values given in Table 2 is plotted against the molecular volume (Table 1) for the cycloalkanemethanols (*circles*) and 1-*n*-alkanols (*triangles*) (data from Ref. 14). *Closed symbols*, active agents; *open symbols with arrows*, highest concentrations of inactive agents examined. The *lines through the closed points* are from linear regressions, except that the point for cycloundecanemethanol was omitted.

[1] J. K. Alifimoff and K. W. Miller, unpublished observations.

Å and 135 Å^3, respectively). However, the observation that 2-propanol (2.5 Å and 127 Å^3, respectively) is an inhibitor[2] resolves the issue in favor of a minimum volume rather than a minimum length being essential for inhibition of nAcChoRs by alkanols, because methanol and ethanol have lengths of 1.5 and 2.4 Å, respectively, and volumes of 67 and 97 Å^3, respectively. Thus, the upper and lower boundaries of inhibitory activity may be related to a maximum and minimum molecular volume, respectively, possibly at a single site. Because propanol is smaller than organic cations, including agonists, that are known to pass through the channel (22), the mechanism for inhibition of ion flux is unlikely be simple channel "plugging" or occlusion, despite the immediate steric indications that this might be so. Such an inhibitory site could be located anywhere along the apparatus linking the agonist site and the channel.

Comparing the ability of an agent to enhance apparent affinity for channel activation (SC_{50}) for cycloalkanemethanols and 1-*n*-alkanols (Fig. 4) with molecular dimensions, it can be seen that the high molecular weight cut-off for flux enhancement correlates well with molecular length. Thus, the cut-off occurs between cyclooctanemethanol and cyclononanemethanol (with lengths of 6.3 and 6.7 Å, respectively) in the cycloalkanemethanol series and between butanol and pentanol (with lengths of 5.0 and 6.3 Å, respectively) in the 1-*n*-alkanol series. There is no equivalent correlation with molecular volume; hexanol, with a volume of 210 Å^3, does not exhibit flux enhancement, whereas this effect is observed with cyclooctanemethanol, which has a volume of 250 Å^3. The fact that cut-off for flux enhancement displays different structural requirements, compared with those for inhibition of ion flux, adds further support to our previous conclusion that these two actions are mediated by different sites.

Nature of the Alkanol Sites of Action

Physicochemical considerations. In the above section we focused upon the steric factors that define the point of cut-off for each effect. These steric factors reflect the underlying repulsive forces of the alcohols that define their shape. In this section we are concerned with the relative potencies of the active agents, which are generally related to the attractive forces between the alcohols and their sites of action except close to the point of cut-off. Such forces increase as methylene groups are added because the intermolecular dispersion forces are roughly proportional to surface area or volume of the alcohol. Indeed, because of this there is a good correlation of IC_{50} (Fig. 3) and SC_{50} with volume (data not shown). A more conventional way to express this is to examine the change in free energy per additional methylene group, a quantity that many have used to compare functional effects with other physicochemical processes to define the nature of the sites of action of the compounds. Such an analysis is presented in Table 4 for various actions of the 1-*n*-alkanols and cycloalkanemethanols reported in the literature.

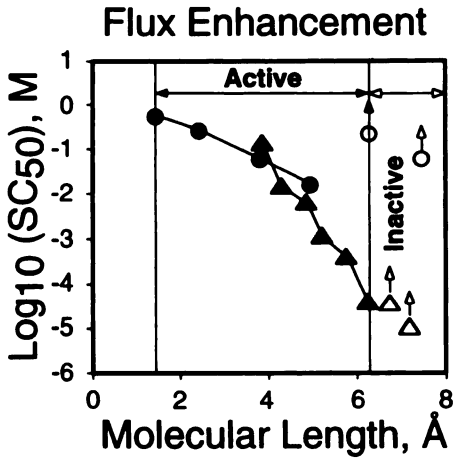

Fig. 4. The cut-off in the ability of alcohols to increase the apparent affinity of agonists for stimulating cation flux through the ion channel of the nAcChoR correlates with their length. The logarithm of the SC_{50} values given in Table 2 is plotted against the molecular length (Table 1) for the cycloalkanemethanols (*circles*) and 1-*n*-alkanols (*triangles*) (data from Ref. 14). *Closed symbols*, active agents; *open symbols with arrows*, highest concentrations of inactive agents examined. The *lines* join the *closed points*.

[2] J. K. Alifimoff and K. W. Miller, unpublished observations.

Previously, we noted that the dependency on the number of methylene groups of the flux-enhancing effect of 1-*n*-alkanols parallels those of their membrane-disordering effects, octanol/water partition coefficients, and general anesthetic potencies, whereas their inhibitory potencies increase more rapidly (11, 14) (Table 4). However, in the cycloalkanemethanol series neither the flux-enhancing action nor the inhibitory action on the nAcChoR parallels either the oleoyl alcohol/water partition coefficients of the compounds or their general anesthetic potencies. There are not yet any data for inhibition of luciferase by cycloalkanemethanols, but in the 1-*n*-alkanol series the free energy enhancement on addition of a methylene group varies considerably along the chain (3), completely unlike the behavior with flux inhibition. In comparing the two series of alcohols, it is clear that both sites of action are nonpolar, but addtional model systems need to be studied before the nature of the sites may be resolved on the basis of free energy analysis.

Approach to the cut-off point. Often nonlinear effects from which mechanistic information can be deduced are observed in the change in free energy per additional methylene as the cut-off point is approached. For example, for general anesthesia with 1-*n*-alkanols the linear increase in potency with number of methylene groups declines only slightly before the cut-off point (23), whereas in the cycloalkanemethanol series the last fully active agent, cyclododecanemethanol, has a potency half that of cycloundecanemethanol (15). A different pattern is seen with firefly luciferase, where the IC_{50} values of the last four or five active 1-*n*-alkanols are very similar but the saturated solubilities of the compounds in the aqueous phase fall with increasing number of methylene groups until cut-off occurs, when the two parameters converge at hexadecanol. It was proposed that these additional methylene groups remain in the aqueous phase and do not contribute to binding (3). In both alcohol series, lipid disordering at equal anesthetic concentrations in a membrane is constant up to a certain point in each series and then declines steadily (15, 24).

When the approach to the cut-off point of the two alcohol series for flux inhibition at the nAcChoR is considered, the observed behavior is most similar to that seen for general anesthesia (Fig. 3). The potency of the cycloalkanemethanols increases smoothly to cyclodecanemethanol but, when one more methylene group is added, instead of the expected decrease in free energy of −3.2 kJ/CH_2 one finds an increase of 8.8 kJ/CH_2, suggesting an unfavorable contribution of 12 kJ/CH_2. If

TABLE 4
The free energy change per methylene group for various properties of the 1-*n*-alkanols and the cycloalkanemethanols

Property	Subgroup	1-*n*-Alkanols		Cycloalkanemethanols	
		C_n[a]	ΔG[b]	C_n	ΔG
			kJ/mol		kJ/mol
Flux[c]	IC_{50}	3–10	−3.8 ± 0.21	4–11	−3.2 ± 0.15
	SC_{50}	1–4	−2.7 ± 0.24	4–9	−3.8 ± 0.24
Anesthesia[d]	Tadpoles	1–10	−3.1 ± 0.12	4–12	−2.69 ± 0.096
Partition coefficient[e]	Octanol/water	1–14	−3.0		
	Oleoyl alcohol/water			4–9	−2.7 ± 0.24
Order parameter[f]	nAcChoR membranes	1–8	−3.1 ± 0.10		
Luciferase[g]	Firefly	1–10	−3.2 ± 0.19		
Cytochrome oxidase[e]	Bovine heart	1–14	−2.4 ± 0.13		
Solubility in water		4–16	−3.2 ± 0.04	5–13	−3.2 ± 0.17

[a] C_n denotes the number of carbon atoms over which the linear regression was made.
[b] ΔG denotes the free energy change per additional methylene group. Values are means ± standard errors.
[c] This work and Ref. 14.
[d] Refs. 15 and 23.
[e] Sources quoted in Ref. 26.
[f] Ref. 11.
[g] Ref. 3. The average free energy enhancement on addition of a methylene group is shown, but unlike the other examples in this table, it varies considerably along the chain.

one assumes a site on a protein, the additional methylene does not simply remain outside the site in an aqueous environment, as with firefly luciferase (3), because if this were the case the potency of cycloundecanemethanol would be comparable to that of cyclodecanemethanol. A simple hypothesis consistent with the data is that a hydrophobic slot containing a buried hydrogen-bonding site exists that is initially wide enough to accept the cycloalkanemethanol ring. However, on addition of the 11th methylene group no low energy conformation of the alcohol can be found that enables cycloundecanemethanol to bind without incurring unfavorable rearrangements of amino acid side chains and/or loss of the hydrogen bond, changes that offset the additional binding energy arising from the dispersion forces of the extra methylene group. As a consequence, inhibitory potency is dramatically reduced. Higher homologs experience even more severe steric hindrance and the net free energy of binding becomes positive.

In the case of flux enhancement, on the other hand, the free energy change per methylene group is unchanged up to the point of cut-off in both series. A consistent hypothesis could be that hydrogen bonding occurs at the mouth of a hydrophobic crevice and additional methylene groups must be placed deeper in the crevice. Beyond a certain length the alcohol can be accommodated in the crevice only at the prohibitive cost of losing a hydrogen bond. On the other hand, plausible lipid models can be constructed that take into account changes in intrinsic curvature (25) that are induced only by such short alcohols.

Comparison with *In Vivo* Action

Direct comparison between the pharmacology for inhibition of the acetylcholine receptors and that for general anesthesia is complicated by the differences in species and temperature. Overall, the cycloalkanemethanols up to cyclodecanemethanol caused general anesthesia in tadpoles (15) at concentrations close to those that inhibit half of the acetylcholine receptor channels. However, cut-off occurs for channel inhibition earlier in the series than it does for general anesthesia for both the cycloalkanemethanols and the 1-*n*-alkanols. Thus, the higher members of both series can cause anesthesia at concentrations that do not lead to channel inhibition. Furthermore, the Hill coefficient for channel inhibition is so low that active homologs inhibit only approximately half of the acetylcholine-activated channels even at the highest physiological concentrations. Although we can conclude that general anesthesia is unrelated to channel inhibition, it would be premature to conclude that acetylcholine receptors are not involved in anesthesia, because alcohols also cause receptor desensitization and the cut-off position for this action has not been published.

Conclusions

The cycloalkanemethanols, a novel series of anesthetic alkanols, have been used in this study as a pharmacological tool to determine the size constraints on the actions of alkanols at the nAcChoR. Both the flux-enhancing and -inhibitory effects display thermodynamic properties that are consistent with interaction at a hydrophobic site. However, in comparing data across the two series, it is apparent that the structure-activity relationships for inhibition of ion flux suggest that this action is mediated via a site of defined volume, whereas those for flux enhancement suggest a site in which molecular length is the limiting parameter. The differing thermodynamic and structural requirements for channel inhibition and flux enhancement are additional evidence that these two actions are indeed mediated by different mechanisms and sites. The specific nature of these sites is not revealed by physicochemical analysis of the available data; this will probably require a more direct approach that the abundance of the *Torpedo* nAcChoR renders uniquely possible.

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Send reprint requests to: Keith W. Miller, Department of Anesthesia, Massachusetts General Hospital, Boston, MA 02114.