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Alkanol effects on early potassium currents in *Aplysia* neurons depend on chain length

(alcohol/ethanol/1-butanol/1-hexanol/anesthesia)

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ABSTRACT **The relationship between alkanol chain length and effects on the transient potassium current, I_A, were examined in three identified *Aplysia californica* neurons with ethanol (EtOH), butanol (BuOH), and hexanol (HxOH). Qualitative differences were found when the actions of EtOH were compared with those of the longer-chain-length alcohols. Whereas EtOH primarily affected the decay time constant of I_A, having minimal effects on amplitude, BuOH and HxOH exerted their major effect on the amplitude of I_A, reducing it, while their effects on decay kinetics were much less pronounced. The effects of EtOH on I_A decay are cell specific among identified neurons of the *Aplysia* nervous system. The actions of BuOH and HxOH did not mimic these interneuronal differences. These data, coupled with data previously reported by us and others, make it unlikely that EtOH exerts its actions on I_A via perturbation of a bulk lipid phase within the membrane.**

The advantages of using alkanols to study the actions of anesthetics include their structural simplicity, which has allowed extensive physical chemical work to be done with them, and the availability of members forming a homologous series of increasing chain length. The correlation of anesthetic potency with lipid solubility, both of which increase with alcohol chain length, has lent support to lipid theories of anesthesia (1). These theories postulate that the effect of the anesthetic molecule is to perturb the membrane lipid environment, secondarily disrupting the function of membrane proteins. A wide range of processes, in addition to anesthesia, show greater sensitivity to alcohols as alcohol chain length increases. Electrophysiologically, this correlation has been seen for voltage-dependent conductances (2–5) as well as for decay of transmitter-evoked currents (6). As the chain length of the alcohols tested is increased beyond a certain length, the anesthetic potency of the alkanols show a relatively sharp cutoff, beyond which longer-chain alcohols are no longer anesthetic. This cutoff has been reported to occur at chain lengths of 8–12 carbons (1, 7–9). The cutoff phenomenon has been theorized by different workers to be compatible with either lipid theories (7) or theories proposing a protein target for the anesthetic molecule (10, 11). A number of membrane phenomena, such as the transition temperature of artificial phospholipid membranes (12, 13), and the inhibition of firefly luciferase by alkanols (10) show this cutoff phenomenon with respect to the actions of alkanols. For some alcohol effects, such as those associated with enzymes involved with cyclic nucleotide regulation, the cutoff has been reported to occur at short chain lengths, at the transition from propanol to butanol (11). In this paper, we examine the relative effects of ethanol (EtOH), butanol (BuOH), and hexanol (HxOH) on the early transient potassium current, I_A, in identified *Aplysia* neurons.

We report that there is not a monotonic dependency on chain length for the actions of these alcohols on I_A, as might be expected if they were acting on a common hydrophobic site. The effects on I_A are qualitatively different for the different chain length alcohols. Also, the variations seen in the actions of EtOH on I_A in different cells (14) are not evident for the longer-chain alcohols. Thus, EtOH at high concentrations may act on I_A via a different mechanism than the longer-chain alcohols. This finding could be of importance in the interpretation of molecular and cellular studies attempting to determine the mechanisms underlying the actions of ethanol on nerve cells, since a large number of these studies use EtOH concentrations greater than 200 mM.

MATERIALS AND METHODS

Aplysia californica weighing between 50 and 100 g were obtained from the Howard Hughes Medical Institute mariculture facilities in Woods Hole, MA. The animals were maintained in artificial seawater (ASW; Instant Ocean, Aquarium Systems, Eastlake, OH) at 15°C. Experiments were performed on the metacerebral giant cell (MCC), the giant buccal cell (B1), and cell R15 in the abdominal ganglion. The results reported for cell B1 were also seen in cell B2 (for cell nomenclature, see refs. 15–17). Ganglia were desheathed by microdissection over the cell to be penetrated. Axotomy of the cell was performed by cutting the axon 150–300 μm from the cell body, to obtain an optimal space-clamp of the cell. Success of the axotomy was verified under voltage clamp by the loss of the late, fast transient currents arising from unclamped axon (18). The ganglion was pinned to the Sylgard-covered bottom of a flow chamber that allowed superfusion with different solutions. The chamber volume was 5 ml, and flow rates allowed the turnover of 5 ml of solution in 3 min.

Selected cells were impaled with two shielded microelectrodes (19), one for recording membrane potential and the other to pass current. Electrodes had resistances between 1 and 4 MΩ (measured in ASW) and were filled with 3 M KCl. Membrane voltage was measured differentially with respect to an extracellular electrode independent of bath ground. The voltage clamp unit used in these experiments (Dagan, model 8500) was extensively modified to achieve a shorter settling time. In the worst cases, the current trace was 95% settled within 750 μsec.

Correction for leakage was made by using a P/2 procedure (20). Data were digitized every 100 μsec and stored on a PDP 11 computer for analysis off-line. After digitization and appropriate scaling, the corresponding current records were algebraically added to correct for leakage. The computer was

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Abbreviations: EtOH, ethanol; BuOH, butanol; HxOH, hexanol; ASW, artificial seawater.

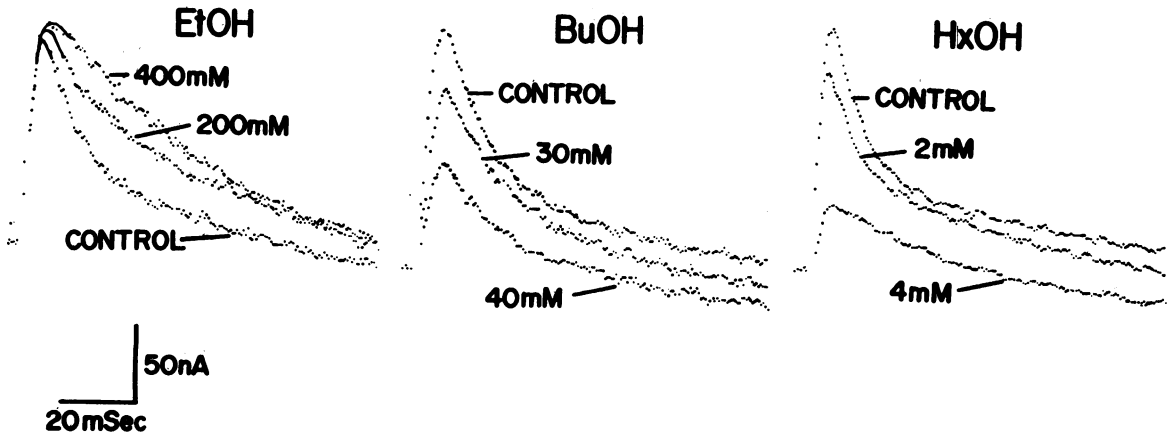

FIG. 1. I_A traces from three R15 cells exposed to increasing concentrations of EtOH, BuOH, or HxOH. The peak currents for the EtOH traces have been highlighted to differentiate them from each other. The currents were elicited by command steps to −20 mV, from a holding potential of −70 mV.

programmed to establish the magnitude of the peak current, the time-to-peak current, and the time constant of current decay. The decay time constant was computed by linear regression using the method of least squares. Multi-exponential decay of I_A in *Aplysia* neurons has been reported (21). We observed a multi-exponential decay of I_A on occasion, but we did not use these cells for this study. We also discarded cells in which I_A did not decay to baseline levels. In the vast majority of cases the r value of the linear regression was greater than 0.99, and it was never less than 0.98 for the decay of I_A during the 100 msec after the peak. All experiments were performed at room temperature (≈20°C). Data are presented as the mean value ± SEM.

The composition of the ASW was as follows: 460 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 55 mM $MgCl_2$, and 10 mM Tris·HCl, adjusted to pH 7.8. To study I_A in isolation from competing currents, the sodium in ASW was replaced with equimolar tetramethylammonium to eliminate sodium currents. Inward calcium currents and outward potassium currents were blocked by adding 125 μM cadmium chloride and 70 mM tetraethylammonium chloride to the perfusion medium. The successful blockade of competing currents with this

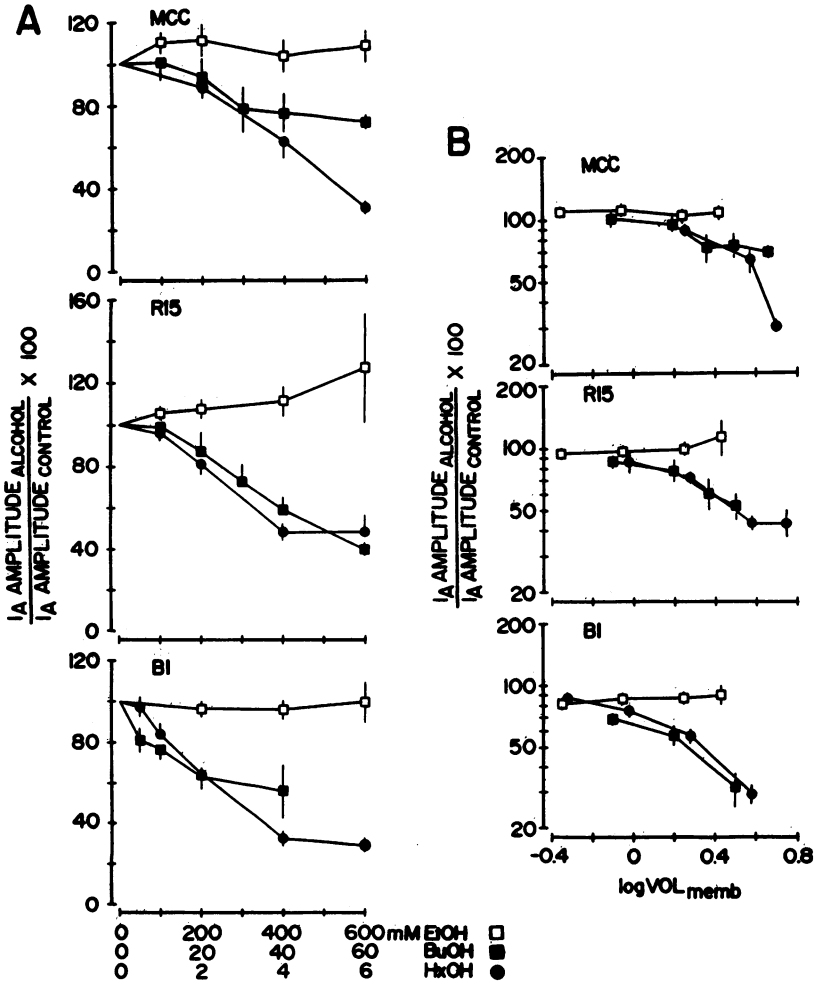

FIG. 2. (*A*) I_A relative amplitude vs. concentration of EtOH, BuOH, or HxOH in each of the three identified neurons examined. (*B*) Same data plotted as a function of the computed membrane volume occupied by alcohol molecules (in ml/kg of membrane; see *Materials and Methods* for description of computations). Each point determination was based on measurements from a minimum of 3, and as many as 13, different cells. Vertical lines indicate SEM.

perfusion medium is demonstrated in ref. 22. Alcohols used in this study were 100% ethanol (U.S. Industrial Chemicals, Tuscola, IL), 1-butanol (Sigma), and 1-hexanol (Sigma). All were of high purity and specified to be free of drying agents or other additives. Hexanol solutions were sonicated immediately before perfusion to ensure dispersion. Series resistance was not compensated, but the maximum error expected for the I_A observed (series resistance value of 10^4 Ω used for computation; ref. 23) was 2.5 mV. EtOH effects reported in this paper could not be accounted for by series resistance error.

Membrane volume occupied by alcohol molecules was computed from the aqueous concentrations of the alcohol, following procedures and using values contained in ref. 1. Briefly, in this procedure, the alcohol concentration within the membrane is determined from observed values of the alcohol buffer/excitable membrane partition coefficient, and the volume of alcohol within the membrane is then derived from the product of the membrane concentration and the molecular volume of the alcohol molecule. A more detailed discussion of this method and the source for values used for partition coefficients and molecular volumes may be found in the review by Seeman (1).

RESULTS

I_A records from R15 are shown in Fig. 1, before and after exposure to the three alcohols tested. All of the effects of each of the alcohols discussed in this paper were reversible by washout of the alcohol.

Effects on I_A Amplitude. Of the I_A parameters tested with BuOH and HxOH, amplitude was the most sensitive. As we have previously reported, EtOH does not have a significant or consistent effect upon I_A amplitude (14, 22). Fig. 2*A* plots the relative amplitude of I_A in each of the three cells in the presence of the three alcohols at various concentrations. Fig. 2*B* plots the same data somewhat differently, with the relative amplitude plotted on a logarithmic scale vs. the logarithm of the computed volume occupied by the alcohol in the lipid phase of the membrane. The strong effect of the longer-chain alcohols on amplitude is apparent. When the data are plotted as a function of molecular volume, it is apparent that BuOH and HxOH have roughly equivalent effects on I_A amplitude when occupying similar molecular volumes, although HxOH is more potent than BuOH in MCC at the highest concentrations. EtOH, on the other hand, does not cause similar reductions in amplitude at the same molecular volumes.

Effects on I_A Decay Time Constant. We have previously reported that EtOH greatly increases the time constant of I_A in most cells (including MCC and R15) but that I_A in some cells (such as B1) shows much less sensitivity (14). The effects of BuOH and HxOH on I_A decay are different from those produced by EtOH. Plots of relative I_A decay time constant vs. alcohol concentrations are shown in Fig. 3*A*, while the data are plotted as relative I_A decay time constant on a logarithmic scale vs. log of molecular volume in Fig. 3*B*.

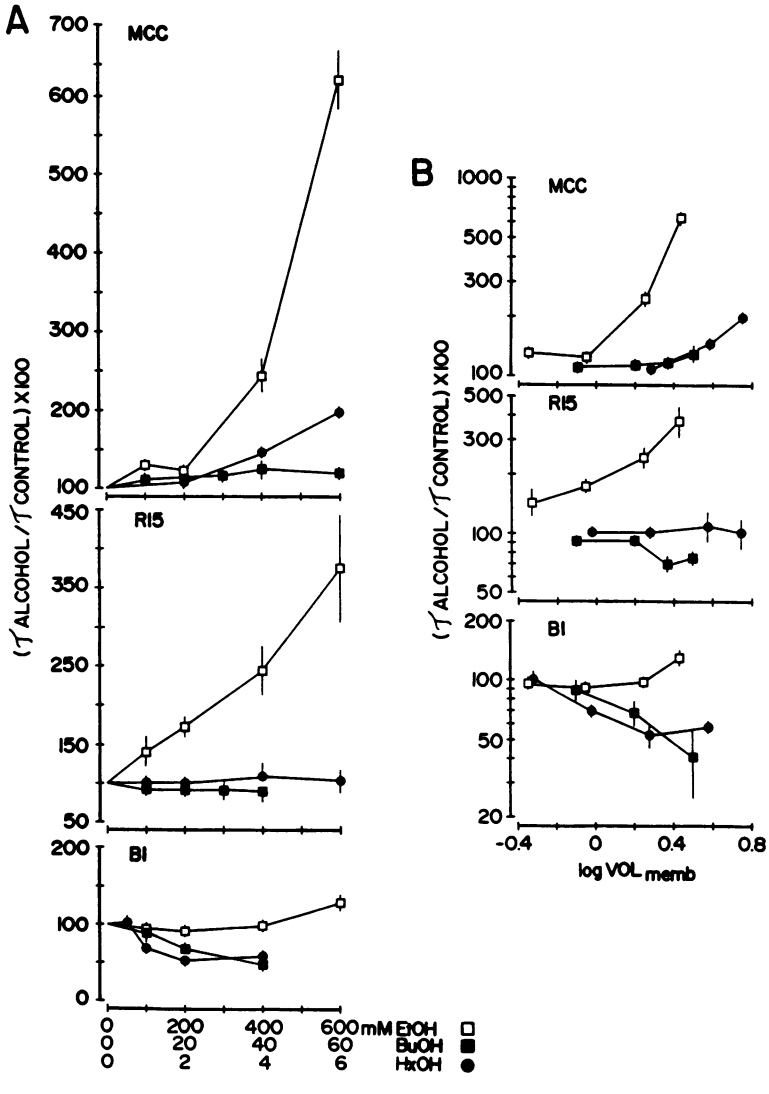

FIG. 3. (*A*) Relative decay time constant (τ) of I_A vs. the concentration of EtOH, BuOH, or HxOH in each of the three identified neurons examined. Each point determination was based on measurements from 3–11 different cells. (*B*) Same data plotted as function of the computed membrane volume occupied by the alcohol molecules (in ml/kg of membrane).

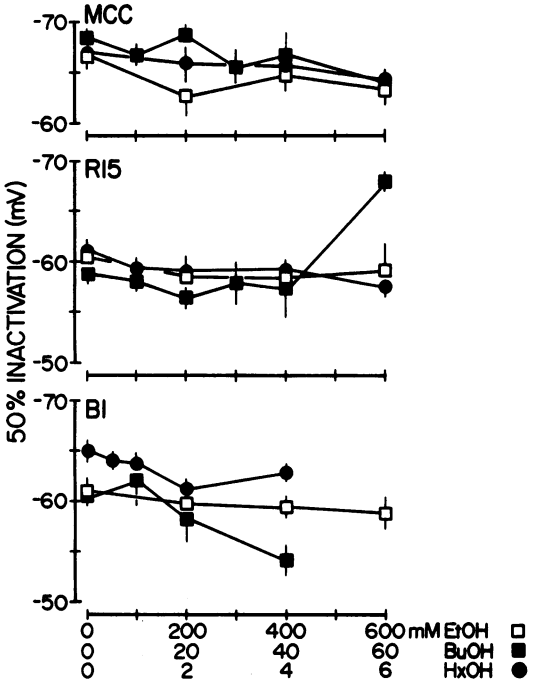

FIG. 4. Prior pulse voltage at which the I_A elicited by a subsequent test pulse to −20 mV was inactivated by 50%, in the presence of EtOH, BuOH, or HxOH. Prior pulse duration was 500 msec. Each point determination was based on measurements from 3–15 different cells.

Both BuOH and HxOH speed up the decay of I_A in B1, and the speedup is approximately equivalent for the two alcohols at equivalent molecular volumes. In MCC, HxOH produces a small increase in decay time constant, although the increase is far less than that produced by EtOH. Neither BuOH nor HxOH influences decay kinetics in R15. Thus, in addition to differences in the most sensitive I_A parameter, the relative sensitivity of I_A among the three identified cells is different for EtOH than for the longer-chain alcohols. The observed changes in decay kinetics produced by BuOH and HxOH were, in all instances, much smaller than the changes produced by EtOH in EtOH-sensitive cells. Inactivation of I_A is the most sensitive of the I_A parameters for EtOH, whereas for the longer-chain alcohols, amplitude is most sensitive.

Effects on Voltage Dependency of Inactivation. I_A is inactivated at membrane potentials more depolarized than the resting potential of the cell (24). We examined whether the three alcohols studied shifted the voltage dependency of inactivation. In Fig. 4 we have plotted the membrane voltage at which a 500-msec pulse produced a 50% reduction in the amplitude of I_A during a subsequent test pulse. As previously reported (14), the three cells tested differ in the control prior pulse potential that produces 50% inactivation. The prior pulse voltages required to produce 50% inactivation were progressively more depolarized for R15, B1, and MCC, in that order. None of the alcohols has a consistent effect on the voltage dependency of inactivation at the concentrations tested (14).

Activation Kinetics and Voltage Dependency of Activation Were Unaffected. We have previously reported that there is a small increase in the time-to-peak I_A produced by EtOH at high concentrations (22). There is no discernible change in the activation kinetics of I_A in any of the three cells with BuOH or HxOH exposure at the concentrations used here. To measure the voltage dependency of I_A activation, the amplitude of current evoked by voltage steps between −40 and −20 mV in 5-mV increments was normalized to the current evoked by steps to −20 mV (see ref. 14 for details of this protocol). EtOH, BuOH, and HxOH were all without effect on the voltage dependency of activation of I_A.

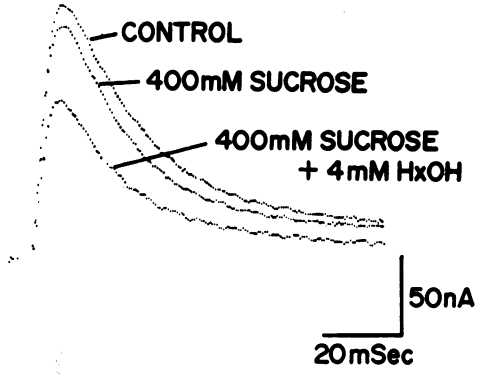

FIG. 5. I_A elicited by a command step to −20 mV in an R15 in the presence of hypertonic sucrose and with HxOH added to the sucrose solution. The results confirm that alterations in osmotic strength of the bathing medium are not responsible for the changes in I_A produced by the alcohols.

Differences in Actions Are Not Due to Osmotic Effects. Since EtOH was tested at much higher aqueous concentrations than either BuOH or HxOH, to obtain equivalent membrane concentrations, the greater osmolarity of the EtOH solutions might account for differences seen among the alcohols. To test this, we monitored I_A in MCC during exposure to physiological medium supplemented with 400 mM sucrose, and then after the addition of 4 mM HxOH to this solution. The results, shown in Fig. 5, indicate that raising the osmolarity of the solution does not mimic the effects of EtOH and that HxOH produces its normal effects when added to the hyperosmotic medium. It may be noted that osmotic effects produced by the alcohols would be transient, since they are relatively permeant and would equilibrate across the membrane.

Intercellular Variability in Alcohol Sensitivity Is Dependent Upon Alcohol Chain Length. The effects of EtOH on I_A are strikingly different in different cells. The decay time constant is strongly affected in some cells, such as MCC and R15, and almost unaffected in other cells, such as B1. The same cell–cell differences are not apparent for the longer-chain alcohols. A direct comparison of the variability of the response of these cells to the alcohols is not possible because EtOH primarily affects decay kinetics, while the parameter most strongly affected by BuOH and HxOH is amplitude. I_A amplitude did not show significant variability in sensitivity to BuOH or HxOH among the three cells studied.

DISCUSSION

EtOH differed from BuOH and HxOH in its effects on I_A in *Aplysia* neurons. The differences were profound and of a qualitative nature. At equivalent membrane volumes, the predominant action of EtOH was to retard the decay of I_A, whereas with both BuOH and HxOH the predominant effect on I_A was to reduce the amplitude. All of these effects were reversible by washout of the drug, suggesting that I_A channels were neither degraded nor removed from the membrane. In addition to their different actions, the intercellular differences apparent for the action of EtOH on I_A were not the same for the actions of either BuOH or HxOH.

The differences between the effects on I_A of EtOH, compared to those of BuOH and HxOH at similar membrane volumes, argue against their acting at a common site and by a common mechanism. The membrane volumes calculated for each of the alcohols at various concentrations were based upon measurements of partition coefficients obtained in non-*Aplysia* membranes and could conceivably be inappro-

priate. However, the fact that the effects of EtOH differed from those of BuOH and HxOH in a number of qualitative ways rather than in a purely quantitative manner makes it unlikely that the divergence of effects seen in the plots contained in this paper result from this type of artifact. Thus, a simple model based upon perturbation of a bulk lipid phase, in which we would expect that at similar membrane concentrations the effects of different alcohols would be equivalent, is not tenable. We have reported that the effects of EtOH on the inactivation of I_A differ, depending upon whether or not the I_A channel population is activated, prior to inactivation, and this suggests that there may be a direct interaction between EtOH and the I_A channel (14). A comparison of the effects of the alcohols tested with the effects of increasing temperature is instructive, and it makes it further unlikely that a bulk lipid phase within the membrane serves as the primary target for alcohol's effects on I_A. Whereas the decay time constant for I_A decreases as the temperature increases (24, 25), presumably concurrent with increasing membrane fluidization, EtOH exposure increases the decay time constant, and the longer-chain alcohols show no indication of decreasing the decay time constant. The amplitude of I_A increases with temperature (S.N.T., unpublished results), and we report here that EtOH has little effect on I_A amplitude and that the longer-chain alcohols reduce I_A amplitude. The lack of correlation between temperature and alcohol results must be interpreted with care, in light of results that we have obtained by using fluorescence photobleaching techniques on *Aplysia* neurons (26). In these studies, the effects of alcohols on the diffusibility of lipid probes were probe specific. In addition, we found that the diffusion coefficient for certain probes was temperature independent, although the proportion of probe free to diffuse increased with increasing temperature, suggesting that a membrane pool of viscous lipid might be recruited to maintain constant fluidity. Thus, although our results argue against the alcohols acting by perturbation of a bulk fluid lipid phase within the membrane, they may be consistent with domain-specific actions within a heterogeneously organized lipid environment. Other alternatives include actions directly on the channels themselves, with specificity of action determined by chain length. It is certainly possible that at high concentrations EtOH exerts a dual effect, both acting on particular lipid domains and interacting directly with I_A channels. Recently, different forms of I_A channel have been reported to occur in insect muscle and nerve (27), and perhaps these different I_A species vary in their response to alkanols. Since we selected cells with single exponential decays and no residual I_A for our studies, it is probable that we are dealing with a uniform I_A population. If we were dealing with two populations, our interpretation would be unchanged, since our results make it unlikely that any of the underlying channel types are responding in a monotonic fashion with respect to alkanol chain length. Our experiments were performed on intact cells, and the actions of metabolites formed from the different alcohols must also be considered as a possibility for the differences in action of the different-chain-length alcohols, although the effects reported in this paper were generally well established within 10 min of exposure to the alcohol. The longer-chain-length alcohols might differ from ethanol in their secondary effects, such as the activation of second messenger systems, which in turn affect channel function. Effects due to alteration of membrane surface charge are unlikely, since there was little evidence of shifts of voltage dependency for either activation or inactivation, with any of the alcohols tested.

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