The effects of short-chain alcohols (methanol, ethanol and n-propanol) on the fast-inactivating, A-type, potassium current of *Lymnaea* neurons were examined using macroscopic recording techniques. Alcohols produced a blockade of the current and modified its inactivation mechanism. The extracellular concentrations of methanol, ethanol and n-propanol causing 50% suppression of the current were 2970, 830 and 230 mM, respectively. The main effects of alcohols on inactivation were a decrease in the amplitude of the slow component and a simultaneous increase in the amplitude of the slow component of inactivation. In a model, the suppression of the fast component could be reproduced by an increase of the backward rate constant related to the dissociation of the inactivation particle from its binding site. The blockade and modification of inactivation reveal similar dependences on ethanol concentration, indicating that the same type of interaction of ethanol with the channel underlies both of these events. Ethanol was effective only in extracellular applications. The data support an action of alcohols at a hydrophobic site near the extracellular portion of the channel.

Alcohols affect the nervous system at different structural levels, including pacemaker activity of individual neurons (Deitrich et al., 1989; Franklin and Gruol, 1987; Silver and Treistman, 1982). Many of these effects are associated with changes in the properties of the ionic channels of different types of cells (Covarrubias and Rubin, 1993; Moore et al., 1990; Treistman et al., 1991; Wood et al., 1991). Much attention has been paid to the effects of ethanol on transient potassium currents or A-currents (Anantharam et al., 1992; Treistman et al., 1991; Treistman and Wilson, 1987a,b), causing a change in the firing rate (Silver and Treistman, 1982). It has been shown that A-currents in different neurons respond differently to ethanol. In *Aplysia* neurons, large concentrations of ethanol cause a decrease in the inactivation rate and an increase in the current amplitude (Treistman and Wilson, 1987a). However, similar concentrations of ethanol cause a drop in the amplitude of *Shaker K*+ currents (ShB1 and ShA1) (Anantharam et al., 1992). The mechanisms by which ethanol produces such opposite effects on various A-channels are still not clear.

There are two hypotheses explaining the effects of alcohols on membranes (Deitrich et al., 1989; Miller et al., 1986; Roth and Miller, 1986). The “lipid hypothesis” assumes that alcohols produce a nonspecific disordering effect on membrane phospholipids, causing an indirect effect on membrane proteins. The second hypothesis, the “protein theory,” suggests that alcohols interact directly with proteins. It is assumed that the receptors for alcohols are located in the hydrophobic domains of the proteins. The latter hypothesis has been supported in a number of reports (Anantharam et al., 1992; Covarrubias and Rubin, 1993; Franks and Lieb, 1984, 1985; Li et al., 1994; Wafford et al., 1991; Wood et al., 1991). It is also possible that both mechanisms are involved at high concentrations of alcohols.

Earlier we described *I*\_af in *Lymnaea* neurons (Alekseev and Zaykin, 1993; Alekseev and Ziskin, 1995). In many instances this current possesses properties similar to those of other A-type currents. However, we found some specific differences in inactivation that have not been revealed in other types of A-currents. This has presented an opportunity for a comparative study of the mechanisms of drug action on different A-channels. The aims of the present study were to examine interactions of alcohols with the A-channels of *Lymnaea* neurons and to describe the mechanisms of alcohol action using a kinetic model of the channel.

Our results show that, similar to other potassium channels, *Lymnaea* A-channels have a low sensitivity to alcohols. Alcohols block these channels and selectively affect inactivation. Hydrophobic interactions of alcohols with the same binding site of the channel protein or adjacent lipids may be responsible for these effects.

**Materials and Methods**

Pronase (for the isolation of neurons) and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). Methanol and n-propanol were...
from Aldrich Chemical Co. (Milwaukee, WI), and ethanol was from Fluka Chemical Co. (St. Louis, MO).

The experiments were performed on internally perfused neurons from the right and left parietal ganglia of the mollusc Lymnaea stagnalis. Macroscopic currents were recorded using a whole-cell voltage-clamp technique. The methods used for isolation of neurons, intracellular perfusion, current recording and curve fitting followed those described in detail elsewhere (Alekseev, 1992). A-currents were elicited by step depolarizations with preceding hyperpolarizing pulses of $\pm 150$ mV, for a duration of 200 msec. Separation of A-currents from the other currents was done by subtracting traces recorded without prepulses of $\pm 150$ mV. This method allows the determination of A-currents in the applied voltage range from $-60$ to $+30$ mV without contamination with other ionic currents (Alekseev and Zaykin, 1993). The inactivation kinetics at subthreshold potentials (from $-70$ to $-90$ mV) were obtained by using a double-pulse method, i.e., the decay of the peak current was measured in a test pulse as a function of the duration of the preceding pulse (Alekseev and Zaykin, 1993).

The inactivation kinetics of $I_{af}$ for long depolarization steps ($\geq 800$ msec) are well described by the sum of three exponential components (Alekseev and Zaykin, 1993). For shorter depolarization steps ($\leq 200$ msec), we used a two-exponential approximation,

$$h(t) = C_0 + C_1 \exp(-\gamma_1 t) + C_2 \exp(-\gamma_2 t)$$

where $C_0$, $C_1$ and $C_2$ are time-independent, pre-exponential coefficients and $\gamma_1$ and $\gamma_2$ are rate constants of the fast and slow components of inactivation, respectively. Equation 1 provided a good fit to the experimental kinetics. The time course of recovery from inactivation was described by a single-exponential function with a rate constant $\gamma_c$. The results are presented as mean $\pm$ S.E.M.

The internal solution contained 80 mM KCl and 10 mM Tris-HCl (pH 7.3). The external control solution contained 1.6 mM KCl, 2 mM CaCl$_2$, 4 mM MgCl$_2$, and 76 mM Tris-HCl (pH 7.5). When alcohols were added, the effect of dilution of the solutions was counterbalanced by addition of more salts into the medium until the initial concentration was reestablished. The experiments were carried out at 18–20°C.

**Results**

Figure 1A shows the results of one of the experiments obtained with different ethanol concentrations. Ethanol caused a decrease in the amplitude and a change in the decay kinetics of $I_{af}$, producing a crossover of the current traces. This effect occurred at all concentrations of ethanol tested and was not dependent on the membrane potential (fig. 2). All changes in $I_{af}$ seen in the presence of ethanol concentrations up to 660 mM for 15 to 20 min were practically reversible after washout of the drug (fig. 1B). Also, we did not find any notable increase in leakage current. These results indicate that ethanol concentrations up to 660 mM do not influence the cell viability.

The dependence of the peak amplitude of $I_{af}$ on the concentration of ethanol is shown in figure 3. It can be described by the equation

$$III_0 = \left(1 + C_\lambda/K_D^n\right)^{-1}$$

where $I_0$ is the current in the control, $I$ is the current in the presence of alcohol, $C_\lambda$ is the concentration of alcohol in the solution and $K_D$ is the apparent dissociation constant, equal to the concentration of alcohol that produces 50% inhibition of the current. The value of $K_D$ for ethanol that fit best to the experimental data was 830 mM.

Ethanol affected the rates and amplitudes of the fast and slow components of inactivation. For currents elicited at $-20$ mV, the values of $\gamma_1$ and $\gamma_2$ in the presence of 660 mM ethanol were increased by 11 $\pm$ 3% and 16 $\pm$ 7% ($n = 9$), respectively. The effect of ethanol on the amplitudes of the fast and slow inactivation components ($C_1$ and $C_2$) is illustrated in figure 2 (traces at $-20$ mV). The changes in the amplitude of fast inactivation at different concentrations of ethanol are shown in figure 3. As can be seen, the decrease of the amplitude of fast inactivation matched the decrease of the peak current. At concentrations of ethanol of $\geq 1600$ mM, the fast component of inactivation almost entirely disappeared from the current records (fig. 4A).
In a number of experiments we studied the effect of intracellular ethanol on $I_{af}$. For this purpose the cells were perfused intracellularly, for 16 to 20 min, with a solution containing 1000 mM ethanol. As a result of this procedure, the peak amplitude was reduced by approximately 15%, with no substantial changes in the amplitudes of fast and slow inactivation (fig. 4B).

At large negative potentials ($-80$ to $-90$ mV), the effect of ethanol on the inactivation rate was more evident (fig. 5). In the presence of 660 mM ethanol the rate constant of the faster component was increased almost 2-fold, whereas the change in the rate constant of the slow component was small. The relative amplitudes of both components changed little.

Ethanol had a strong effect on the time course of recovery from inactivation (fig. 6). At the ethanol concentration producing 50% suppression of the current (830 mM), the rate constant for the recovery from inactivation was increased 2.2 ± 0.3-fold. This effect did not depend on the potential. Regardless of the ethanol concentration in the external solution (90–1000 mM), we did not find any change in the shape of the steady-state inactivation curve or its position on the voltage axis, within an accuracy of ±5 mV.

In contrast to inactivation, the activation gating apparatus of $I_{af}$ is practically insensitive to the action of external ethanol. Figure 7 shows the effect of ethanol on the current-voltage relationship and activation kinetics of $I_{af}$. The activation kinetics were derived by dividing the measured current values by the inactivation variable $h(t)$ (eq. 1), $I_{at}(t)/h(t)$ (Alekseev and Ziskin, 1995). In this procedure we also excluded the influence of inactivation on the current amplitudes (fig. 7B). At the tested concentrations of 300 to 660 mM, we saw no effect of ethanol on the current-voltage relationship (except the decrease in the amplitude) or on the activation kinetics.
In the presence of 660 mM ethanol, the amplitude of the current obtained from the \( \frac{I_{af}(t)}{I_{h}(t)} \) ratio was decreased 1.51-fold (for data in fig. 7). The main cause of the inhibition of the current amplitude may be a blockade of the \( I_{af} \) channels. Ethanol can block the channel either in the closed states or in the open state. To investigate these possibilities we studied the effect of ethanol on tail currents (fig. 8). After a depolarization pulse of +20 mV, which activated most of the channels, a step repolarization to −90 mV caused a closing of the channels. This resulted in current decay, referred to as a tail current. Ethanol decreased the amplitude of the tail current and slowed its decay rate (fig. 8). The main reason for slowing of the decay rate could be a reopening of blocked channels, which means that the blockade of the channels occurred mostly in the open state. Other possible mechanisms underlying this effect are not obvious, because a direct effect of ethanol on the activation gating apparatus was not observed.

We tested the effects of two other alcohols, methanol and \( n \)-propanol, on \( I_{af} \) (fig. 9). The changes in the current in the presence of these alcohols in the external solution were basically similar to those caused by ethanol. Both alcohols produced a typical crossover of the current traces. The channels of \( I_{af} \) were reversibly blocked by these alcohols, in a concentration-dependent manner. The concentrations of methanol and \( n \)-propanol causing 50% suppression of the current were 2970 and 230 mM, respectively. The steady-state inactivation, the voltage dependence of activation and the activation kinetics of \( I_{af} \) were largely unaffected by these alcohols. In the presence of 230 mM \( n \)-propanol, producing 50% suppression of the current, the rate constant for the recovery from inactivation was increased 2.3 ± 0.3-fold.

**Discussion**

Alcohols produced a decrease in \( Lymnaea \) A-currents and selectively affected inactivation. A number of reports described the alcohol blockade of different ionic channels (Covarrubias and Rubin, 1993; Moore et al., 1990; Mullin and Hunt, 1985; Treistman et al., 1991). In most cases, effective concentrations of short-chain alcohols were high and comparable to those used in this study. The changes in \( Drosophila \) ShB1 currents in the presence of ethanol were in some respects similar to the changes in \( Lymnaea \) \( I_{af} \) (Anantharam et al., 1992). Therefore, the mechanisms underlying the effects of ethanol on these channels may also be similar.

The main effect of alcohols on the inactivation of \( I_{af} \) was the
decrease in the amplitude of the fast component and the simultaneous increase in the amplitude of the slow component of inactivation. These changes were accompanied by an increase in the rate of fast inactivation. At high concentrations of ethanol, the fast component of inactivation almost entirely disappeared (fig. 4). Selective suppression of the fast component of inactivation by alcohols indicates 1) that different mechanisms underlie the fast and slow components of inactivation of $I_{af}$ and 2) two types of inactivation can function separately.

All alcohols tested produced a crossover of the currents. A well-known blocker of potassium channels, TEA, also produces the crossover of the current traces, due to a decrease in the peak amplitude and a substantial slowing of fast inactivation (Alekseev and Ziskin, 1995; Choi et al., 1991). This results from TEA competition with the inactivation particle for the binding site (Choi et al., 1991). The main reason for the crossover of the current traces during alcohol blockade was the increase in the amplitude of the slow component, rather than slowing of fast inactivation. These results indicate that alcohols do not compete with the inactivation par-
particle for the binding site, i.e., the mechanism of the blockade by alcohols is quite different from that by TEA.

It should be noted that alcohols at high concentrations produce large osmotic pressure gradients across the cell membrane. It is of interest whether osmotic pressure affects the current kinetics. In experiments with methanol, we found that methanol induced changes in the current amplitude and inactivation similar to those induced by ethanol, but at concentrations 3.5 times higher than those of ethanol. Because the osmotic pressure is proportional to the alcohol concentration, the cell membrane was subjected to a much higher osmotic pressure in the presence of methanol than in the presence of ethanol. This result shows that the observed changes in the current did not follow the changes in osmotic pressure. Moreover, as was shown for Aplysia neurons, increases in the sucrose concentration in the external solution, up to 400 mM, had little effect on A-currents (Treistman and Wilson, 1987b). It seems that mollusc A-currents have low sensitivity to changes in osmotic pressure.

It has been shown that the solubility of alcohols in lipids increases by a factor of about 3.5 with each additional carbon atom (Lindenberg, 1951; Lyon et al., 1981). Haydon and Urban (1983) confirmed the validity of the logarithmic relationship between the alcohol concentration causing 50% suppression of the sodium current and the number of carbon atoms. Figure 10 shows a similar dependence for \( I_{Na} \) in Lymnaea neurons. A plot of \( \log K_D \) for all three alcohols against the number of carbon atoms is linear, with a slope corresponding to a factor of 3.6. Thus, it can be concluded that the effects of alcohols on \( I_{Na} \) channels correlate with the solubility of alcohols in membrane lipids and, thus, with the content of alcohols in the membrane.

Intracellular ethanol had little effect on the peak amplitude and inactivation of \( I_{Na} \). The \( I_{Na} \) channels are affected by alcohols penetrating into the membrane from the external side. This result supports the hypothesis that aliphatic alco-
hols with chains up to three carbons in length concentrate at the membrane surface, with their OH groups possibly being anchored at the membrane/water interface (Zavoico et al., 1985).

Our data show that the gating apparatus of activation was not affected by alcohols. Only inactivation was changed significantly. Hence, alcohols act selectively on the regions of the channel protein responsible for inactivation, rather than by perturbing the whole structure of the protein. Because the alcohol potency correlates with the alcohol content in neuronal membranes, hydrophobic interactions of alcohols with the channel protein or adjacent lipids seem to play a main role in the effect.

Two main effects of ethanol on the channel, the blockade and the modification of inactivation, reveal similar dependence on ethanol concentration (fig. 3). Hence, both the blockade and the modification of inactivation, although different mechanisms underlie these events, could result from the same type of interaction of ethanol with the channel. One of the possible explanations of this result is that the conformational change in the channel responsible for modification of inactivation may also control the blockade of the channel.

It is well known that voltage-dependent characteristics of ionic currents, such as steady-state activation and inactivation, as well as the activation rate constants are highly sensitive to changes in surface charge (Alekseev and Ziskin, 1995; Gilly and Armstrong, 1982; Hille et al., 1975). The absence of an effect of alcohols on similar characteristics of A-channels in *Lymnaea* neurons shows that short-chain alcohols do not change the surface charge or the surface potential of the membrane.

In a previous report we presented a kinetic model for the *I* _af_ channels, describing the complex inactivation kinetics over a wide voltage range (from −90 to +30 mV) (Alekseev and Ziskin, 1995). To simplify the modeling of effects of alcohols, we chose the part of this scheme that describes the kinetics of this channel in the voltage range from −60 to −30 mV (fig. 11, scheme 1). In this model, inactivation is partially coupled to activation (Zagotta and Aldrich, 1990). The multiple inactivation process is represented by two sets of voltage-independent transitions, O ↔ I 1 and O ↔ I 3 ↔ I 5, describing the fast and slow components, respectively. The additional transitions from I 1 to I 2, I 3 and I 5 were added to satisfy the partially coupling condition for fast and slow inactivation, according to the work of Hoshi et al. (1991). The rate constants of the transitions I 1 ↔ I 2 and I 3 ↔ I 5 were set equal to those of the transitions O ↔ I 4 and I 3 ↔ I 5, respectively. Therefore, in fitting the model to the inactivation kinetics, it was enough to find the rate constants of the three transitions O ↔ I 1 and O ↔ I 3 ↔ I 5. In the potential range of −80 to −90 mV, inactivation was described by the transitions C 3 ↔ I 6 ↔

**Fig. 10.** Dependence of the alcohol concentration required to suppress *I* _af_ by 50% (K_D) on the number of alcohol carbons.

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**Scheme 1**

C → O → 85 → I 1

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**Scheme 2**

C → O → 85 → I 1

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Two main effects of ethanol on the channel, the blockade and the modification of inactivation, reveal similar dependence on ethanol concentration (fig. 3). Hence, both the blockade and the modification of inactivation, although different mechanisms underlie these events, could result from the same type of interaction of ethanol with the channel. One of the possible explanations of this result is that the conformational change in the channel responsible for modification of inactivation may also control the blockade of the channel.

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**Fig. 11.** Kinetic models of *I* _af_ that fit all macroscopic parameters of the currents in control (scheme 1) and in the presence of 660 mM ethanol (scheme 2), in the potential range from −60 to −30 mV. C ↔ O transitions describe activation of the channel from the closed states (C 1 to C 3) to the open state (O), C 1 ↔ C 2 ↔ C 3 ↔ C 4 ↔ O. The values of the voltage-dependent forward and backward activation rate constants a, and b, (n = 1, 2, 3 or 4) are equal to those found earlier (Alekseev and Ziskin, 1995), a i = 4α, a i = 3α, a i = 2α/0.95, a i = α/2.1, b i = β, b i = 2β, b i = 3β, b i = 4β, α = 1500 exp(0.025-V) sec⁻¹ and β = 10 exp(-0.027-V) sec⁻¹, where V is the membrane potential (in mV). The voltage-independent rate constants (sec⁻¹) of the transitions to inactivated states (I) are given in schemes 1 and 2 next to the corresponding transitions. BI represents the set of blocked states of the channel B ↔ BI 1 ↔ BI 2 ↔ BI 3, with the rate constants of each transition equal to those of the corresponding transitions O ↔ I 1 ↔ I 2 ↔ I 3.
$I_v$, with the rate constants equal to those of the corresponding transitions $O \leftrightarrow I_v \leftrightarrow I_{v0}$.

The blockade of the channel by alcohols was best modeled by a transition from an open state to a blocked state (fig. 11, scheme 2). Experimental data show that this blockade does not involve a competition mechanism. Hence, inactivation could also occur from a blocked state. This result was reproduced by including in the blocked state the transitions responsible for inactivation (BI). We cannot know the actual rates for ethanol blocking and unblocking, but simulated current traces were the same as long as unblocking was much faster than inactivation. The blocking potency of ethanol was dependent only on the ratio of blocking and unblocking rate constants.

In both schemes we used the same voltage-dependent rate constants for activation, because the effects of alcohols on activation were insignificant. The calculated magnitudes of the rate constants are shown in figure 11. The fits of schemes 1 and 2 to experimental data are illustrated in figure 2.

Attempts to model the blockade with the transitions from the closed states to the blocked states have not yielded positive results. They have shown a decrease in the inactivation rate at high hyperpolarizing voltages, which contradicts experimental data. Thus, the model shows a high probability of the channel being blocked from the open state.

As can be seen, ethanol modifies some of the inactivation rate constants. The most affected is the backward rate constant of the transition responsible for fast inactivation. This rate constant, which is related to the dissociation of the inactivation particle from its binding site, is increased dramatically (almost 10-fold in the presence of 660 mM ethanol, compared with control). At the same time, alcohol has practically no effect on the forward rate constant of this transition, indicating that the association rate of the inactivation particle is not affected by alcohol. In contrast to fast inactivation, the slow inactivation states are stabilized in the presence of alcohol, causing an increase in the amplitude of the slow component of inactivation.

Fast inactivation of $I_v$ seems to involve a ball-and-chain mechanism (N-type inactivation) (Alekseev and Ziskin, 1995). The probable cause of the selective increase in the backward rate constant of the fast inactivation transition is the destabilization of the inactivation particle-receptor complex due to changes in the receptor conformation. Because external alcohols were more effective in producing this effect, alcohols probably have an allosteric effect on the receptor structure.

The amplitude of the current depends on two factors, i.e., the destabilizing and blocking effects of alcohols. The decrease in the amplitude of the fast inactivation component due to increases in the backward rate constant compensates, to some extent, for the blocking effect of alcohols. The peak amplitude can even increase if the blockade is weak. This result may explain the different effects of alcohols on the amplitudes of different $A$-currents.

Many of the experiments performed in this study used relatively high concentrations of ethanol, even some that exceed lethal levels in intact vertebrates (Deitrich et al., 1989). The high concentrations were necessary to obtain a more complete understanding of the mechanisms by which alcohols interact with membrane proteins. However, we were also able to demonstrate some effects using relatively small concentrations (figs. 1 and 3), which are less than some values observed in alcoholic patients (Berillard and Hasselbalch, 1981). Also, it has been shown that relatively small changes in potassium currents can produce relatively large changes in transmitter release, signal transduction (Caswell and McLachlan, 1986; Klein et al., 1982; Rogawski, 1985) and neuronal firing rates (Connor and Stevens, 1971). The latter may contribute to observed behavioral effects in human alcoholism. It seems that, due to low sensitivity of $A$-currents to alcohols, the changes in $A$-currents do not play a major role in the effects of alcohol on organisms. Some mammalian systems are more sensitive to low concentrations of ethanol and may be the primary targets for alcohol action (e.g., Deitrich et al., 1989; Moore et al., 1990; Wafford et al., 1991). Our study provides insight into the mechanisms of interaction of alcohols with the membrane proteins.

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References


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