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Ethanol selectively blocks a noninactivating K^+ current expressed in *Xenopus* oocytes

(anesthetic action/Shaw potassium channel/whole-oocyte voltage clamp)

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ABSTRACT **There is presently a debate regarding the relative merits of lipid-based and protein-based theories of anesthesia and the action of ethanol in the central nervous system. Voltage-sensitive K^+ channels play a key role as regulators of neuronal electrical activity and are potential targets of ethanol and other anesthetic agents. We investigated the action of low concentrations of ethanol on four structurally homologous cloned K^+ channels expressed in *Xenopus* oocytes. We report that only the *Drosophila* Shaw2 channel, which does not inactivate upon prolonged depolarization, is rapidly and reversibly blocked by ethanol in a concentration-dependent manner (17–170 mM). The concentration dependence of the blockade can be explained by assuming a bimolecular interaction between ethanol and the channel. We also found that Shaw2 K^+ channels were selectively blocked by halothane (1 mM). Our results support the "protein hypothesis" of ethanol and anesthetic action. These findings open ways to elucidate directly the molecular mechanism of interaction between general anesthetics and a voltage-sensitive K^+ channel.**

Ion channels and receptors in the central nervous system have been associated with the acute physiological effects of ethanol (1). For instance, receptor-mediated responses elicited by *N*-methyl-D-aspartate or γ-aminobutyric acid are inhibited and potentiated by ethanol, respectively, and ethanol promotes desensitization of nicotinic acetylcholine receptors (2). Also, voltage-sensitive Ca^{2+} and K^+ channels are affected by ethanol *in vivo* (3–5). Thus, ethanol (and presumably other general anesthetics) can act on multiple targets. However, the molecular mechanism of ethanol action remains unknown and there is debate about the nature of the site of action (1, 6). It has been proposed that ethanol and other anesthetics act on membrane proteins directly or, alternatively, indirectly via perturbation of the surrounding lipids.

K^+ channels represent the most diverse group of voltage-sensitive ion channels and are encoded by a multigene family conserved in invertebrate and vertebrate organisms (7–11). These channels are multisubunit proteins that can function as homo- or heteromultimers (12). The recent cloning of several voltage-sensitive K^+ channels has presented an opportunity to study the structure–function relation and the mechanisms of drug action (14–16). To study the mechanism of ethanol action, we investigated the effect and selectivity of ethanol among closely related cloned K^+ channels expressed in voltage-clamped *Xenopus laevis* oocytes. Since these channels are structurally homologous, and we have expressed them in the same cell, we can assess the importance of the protein moiety in determining the action of ethanol. We found that *Drosophila* Shaw2 K^+ channels were selectively blocked by low concentrations of ethanol, which suggests that this action is specifically associated with the Shaw2 polypeptide.

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MATERIALS AND METHODS

cRNA Synthesis and Electrophysiology. Shaw2, mShal1, and Shaker H37 cDNAs were subcloned in pBluescript II KS (Stratagene). To obtain polyadenylylated transcripts of Shaw2 and mShal1, a $(dT)_{34}$ tail was inserted at the *Not* I site in the polylinker. Kv1 cDNA was subcloned in pGEM-A (17). Capped run-off cRNAs were synthesized from linearized templates of Shaw2, mShal1, Shaker H37, and Kv1. Then, a Nanoject microinjector (Drummond) was used to inject ≈50 nl of cRNA into each defolliculated *Xenopus* oocyte. Electrophysiological recording was done 2–3 days after injection, with standard two-microelectrode voltage clamp (TEV-200, Dagan). The standard recording solution was 96 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/5 mM Hepes/2.5 mM sodium pyruvate, pH 7.4. The volume in the recording chamber was ≈350 μl and, before drug application, the oocytes were regularly perfused with standard recording solution. Ethanol and halothane (diluted in standard recording solution immediately before use) were applied by bath perfusion at 3–4 ml/min (≈10 chamber volumes). To continuously monitor the input resistance of the cell during a recording session (see Figs. 1*B*, 2, and 3), each test pulse was preceded by 20-mV hyperpolarizing and depolarizing prepulses. Changes in input resistance were not associated with ethanol or halothane perfusion. Current traces were corrected by assuming a linear leak. All recordings were done at room temperature (21–23°C) and all traces were low-pass-filtered at 1 kHz and digitized at 2 kHz. Data were acquired and analyzed with PCLAMP (Axon Instruments), QPRO (Borland), and NFIT (Island Products).

RESULTS AND DISCUSSION

Ethanol Blocks Shaw2 K^+ Channels. Ethanol reduced the amplitude of whole-oocyte Shaw2 K^+ currents (Fig. 1*A*), and the onset of the ethanol-induced blockade was relatively rapid (Fig. 1*B*). About 90% of the steady-state block was reached within 30 s of ethanol perfusion (details in Fig. 1 legend). The main effect of ethanol was on the amplitude of the current. The current–voltage relationship was not affected by ethanol (Fig. 1*C*), and the activation kinetics of the whole-oocyte current were only minimally retarded (Fig. 1*D*) (for comparison, maximum current amplitude in the presence of ethanol was scaled to match the control). In addition, the ethanol-induced blockade of Shaw2 K^+ currents was concentration-dependent (Fig. 2*A*) and is well described by a hyperbolic binding isotherm over the studied concentration range (Fig. 2*B*). This interpretation assumes a bimolecular reaction (one-to-one), and implies the presence of a saturable

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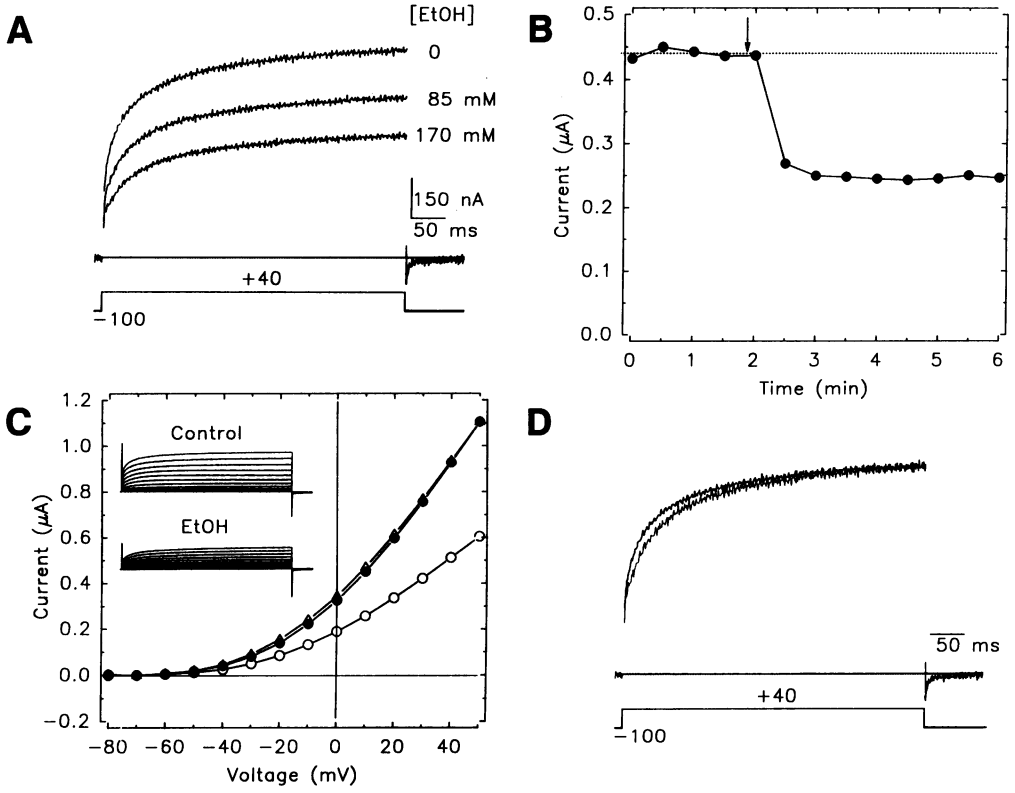

FIG. 1. Ethanol mainly reduces the amplitude of *Drosophila* Shaw2 K^+ currents expressed in *Xenopus* oocytes. (*A*) Whole-oocyte Shaw2 K^+ currents recorded in the absence and in the presence of ethanol (85 and 170 mM). Currents were elicited by 450-ms step depolarizations to +40 mV from a holding potential of −100 mV. For clarity the capacitative transient was blanked by deleting the first sampling point at the onset of the pulse. (*B*) Amplitude of Shaw2 K^+ currents plotted at intervals of 30 s. Currents were elicited by 450-ms test pulses to +30 mV from a holding potential of −100 mV. These currents were recorded at intervals of 30 s, and their amplitude was measured by averaging the last 10 sample points. Ethanol (170 mM) was applied at the time indicated by the arrow. About 90% of the steady-state blockade was reached within the first 30 s of ethanol application. (*C*) Current–voltage relation in the absence of ethanol (●) and in the presence of 170 mM ethanol (○). For comparison, the curve obtained with ethanol was normalized to the amplitude of the control response at +50 mV (△). *Inset* shows the corresponding current traces (900-ms depolarizations). Notice that ethanol reduced only the amplitude; the current–voltage relation was not affected. (*D*) Activation kinetics of the whole-oocyte Shaw2 K^+ current in the absence of ethanol and in the presence of 170 mM ethanol (lower trace). For comparison, the trace obtained with ethanol was normalized to the amplitude of the control trace. The kinetics of the current are minimally affected by ethanol.

site responsible for the blockade of Shaw2 K^+ channels. Also, as expected for a simple interaction between ethanol and Shaw2 K^+ channels, the block of the current was fully reversible (Fig. 2). The studied concentration range of ethanol is equivalent to 80–800 mg/100 ml. Ethanol concentrations on the order of 200–400 mg/100 ml produce anesthesia in alcoholics, and higher concentrations are generally considered lethal (19).

Low Concentrations of Ethanol Do Not Affect Other Cloned K^+ Channels. We also studied three voltage-gated K^+ channels representing the Shal and Shaker subfamilies. mShal1 is a mouse brain homolog of the Shal subfamily (20); Shaker H37 is an alternatively spliced variant of the *Drosophila* Shaker gene (21); and Kv1 is a rat brain Shaker homolog (17). The mShal1 and Shaker H37 channels exhibit rapidly inactivating A-type K^+ currents, whereas Kv1 exhibits slowly inactivating, delayed rectifier-type K^+ currents. Relatively high concentrations of ethanol had little or no effect on the amplitude or kinetics of these K^+ currents (Fig. 3). Another study (22) reported that 10 distinct K^+ channels expressed in *Xenopus* oocytes (those channels are not included in our study) were affected to various extents only by very high concentrations of ethanol (≥200 mM). Thus, among a total of 14 cloned K^+ channels, low concentrations of ethanol (<200 mM) selectively block *Drosophila* Shaw2 K^+ currents expressed in *Xenopus* oocytes. The sensitivity of Shaw2 K^+ channels to ethanol complements other unique biophysical properties of these channels (9, 23). For instance, among all cloned voltage-gated K^+ channels related to Shaker, only Shaw2 channels lack any apparent macroscopic inactivation during a prolonged depolarization. They also show relatively weak voltage sensitivity (Fig. 1*C*), a finding that is consistent with the low number of putative gating charges in the S4 transmembrane segment of the channel polypeptide.

Shaw2 K^+ Channels Are Also Blocked by a Volatile Anesthetic. Extracellular ethanol concentrations on the order of 50–100 mM produce general anesthesia in warm-blooded animals (19), and it has been proposed that in some instances ethanol and volatile anesthetics share a common mechanism or site of action (1, 6). To test this hypothesis, we studied the effect of halothane on Shaw2 channels (ethanol-sensitive) and mShal1 channels (ethanol-insensitive). Shaw2 K^+ currents were reversibly blocked by 1 mM halothane, whereas mShal1 K^+ currents were essentially unaffected by 3 mM halothane (Fig. 4). The effects of ethanol and halothane were qualitatively similar. Thus, these drugs may act on Shaw2 channels by an analogous mechanism. Experiments to test competition between alcohols and halothane may give additional insights.

Selective Blockade of Shaw2 Channels by Ethanol and Halothane Supports the Protein Hypothesis of Anesthesia. Two hypotheses have been used to explain the effects of ethanol and volatile anesthetics on membrane proteins (1, 2, 6): (*i*) indirect interaction mediated by nonspecific disordering of

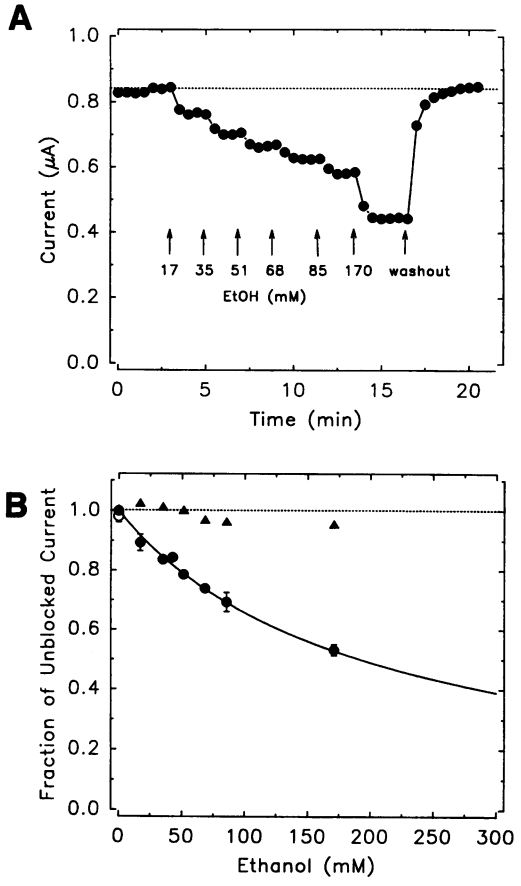

FIG. 2. Ethanol blocks Shaw2 K^+ currents in a concentration-dependent manner and the blockade is completely reversible. (*A*) Current amplitude plotted at 30-s intervals (currents were elicited as described in Fig. 1 legend). The indicated concentrations of ethanol were perfused in the recording chamber at the time marked by the arrows. Standard recording solution was used to wash out the blockade induced by ethanol. The blockade increased as a function of ethanol concentration and it was completely reversible. The concentration range studied here is equivalent to 80–800 mg/100 ml. (*B*) The fraction of unblocked current is plotted versus ethanol concentration. For Shaw2 (●), six oocytes were analyzed and pooled. Each symbol represents the mean ± SD of three to five independent determinations. The maximum size of the current at +50 mV was 0.4–1 μA for all cells included in this analysis. The open circle at zero ethanol represents the normalized amplitude after the washout. The solid line across the points represents the best fit (least-squares) to this equation: $FUB = K_B/(K_B + [B])$, where FUB is the fraction of unblocked current, K_B is the apparent dissociation constant, and [B] is the concentration of ethanol. To fit this equation all individual determinations were included. The value of K_B which produced the best fit was 192 mM. The agreement between the experiment and the theory suggests a simple bimolecular interaction between ethanol and Shaw2 K^+ channels. If the site of ethanol action were located in a hydrophobic domain of the channel in contact with the lipid bilayer, we assume that the relation between the concentrations of ethanol in the aqueous phase and the bilayer would be linearly proportional. Physical measurements indicate that this is a reasonable assumption in the dilute concentration range studied here (2, 18). For Kv1 (▲), only one experiment is represented. This current is essentially insensitive to ethanol.

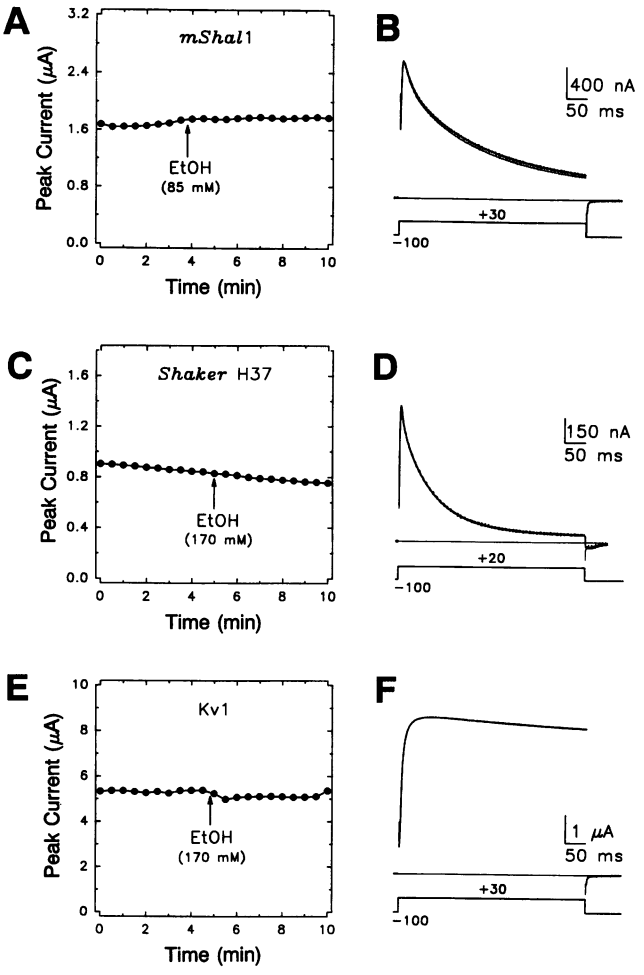

FIG. 3. mShal1, Shaker H37, and Kv1 channels exhibit ethanol-insensitive K^+ currents. Experiments in *A*, *C*, and *E* were carried out as indicated in Fig. 1 legend. Current traces in *B*, *D*, and *F* are sample traces shown to detect changes in the kinetics of the currents. Thus, current traces recorded after ethanol were scaled to exactly match the control peak current and overlaid with a control trace. In all cases, the selected current traces with ethanol were taken at least 90 s after the onset of ethanol perfusion. Only the time course of mShal1 current inactivation (thinner trace in *B*) was minimally affected (170 mM ethanol did not produce a significantly greater effect). The time courses of Shaker H37 and Kv1 currents before and after ethanol are undistinguishable. The voltage-clamp paradigm is indicated at the bottom of each set of traces. Notice that although Shaker H37 peak current runs down at a steady rate (*C*), the time course of rundown is not affected by ethanol.

membrane phospholipids (lipid hypothesis); and (*ii*) direct interaction with membrane proteins (protein hypothesis), which implies a drug–receptor interaction at a site located in a hydrophobic domain of the protein. The relative merits of these hypotheses remain controversial, although evidence has been presented to support the validity of the latter (24–30). The simplest interpretation of our results supports the protein hypothesis, mainly because among several structurally homologous K^+ channels expressed in *Xenopus* oocytes (i.e., in a constant cellular environment), Shaw2 K^+ channels are selectively blocked by ethanol and halothane. To some extent, a combination of the lipid and protein hypotheses (2, 6) may account for our observations. For instance, ethanol or halothane may interact with specific annular lipids that are closely associated with Shaw2 K^+ channels and regulate their function. However, the presence of a discrete saturable site does not support this hypothesis (Fig. 2). Alternatively, volatile anesthetics and ethanol may act through a second-messenger pathway (30). This mechanism is more complex and seems (in our case) unlikely, mainly because other observations in our laboratory indicate that second-messenger-mediated effects on K^+ channels in intact *Xenopus* oocytes display significantly slower onset and kinetics (13).

In this study, we have demonstrated selective blockade of one subtype of cloned K^+ channels by low concentrations of

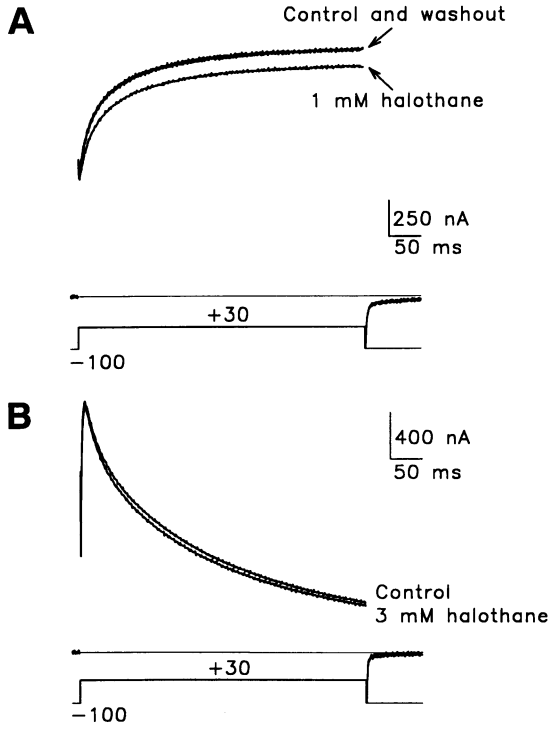

FIG. 4. Halothane blocks the ethanol-sensitive Shaw2 K^+ current. (*A*) Overlaid Shaw2 currents in the absence of halothane (control), in the presence of 1 mM halothane (recorded 90 s after the onset of the application), and after washout. From a saturated stock solution (kept in ice), halothane was diluted at the indicated concentration immediately before application. (*B*) Overlaid mShal1 currents in the absence (control) and in the presence of 3 mM halothane. The ethanol-insensitive mShal1 current was not affected by halothane. The voltage-clamp paradigm is shown at the bottom in both *A* and *B*.

ethanol and by halothane. Our results are consistent with the protein hypothesis of anesthetic action and a direct one-to-one ethanol–channel interaction. Experiments involving mutagenesis of the Shaw2 gene and single channel recording may help to test this hypothesis more directly and possibly reveal the molecular mechanism of anesthetic action on a voltage-sensitive K^+ channel.

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