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Differential Effects of Ethanol on Electrical Properties of Various Potassium Channels Expressed in Oocytes

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SUMMARY

The effects of ethanol on a number of electrophysiological parameters were examined in 10 different voltage-gated potassium channels expressed in *Xenopus* oocytes. None of the channels examined was highly sensitive to ethanol, but there was significant variability among the channels tested at concentrations of ethanol of 200 mM and greater. The response to ethanol was not determined exclusively by membership in a genetic subfamily. In addition, the relative sensitivity among different channels could vary independently for different electrical parameters. For example, current amplitude in DRK1 was insensitive to ethanol, even at concentrations as high as 600 mM, whereas this was one of the more sensitive channels with respect to the kinetics of current inactivation. The opposite situation was true for ShA1. Therefore, ethanol at high concentrations may selectively perturb discrete regions of channel proteins. This is supported by the finding that removal of 318 amino acids from the cytoplasmic carboxyl terminus of DRK1 results in a channel whose current amplitude shows greater sensitivity to ethanol than does DRK1. Thus, the effects of ethanol on the channel may not be limited to interactions at the lipid-protein interface.

Ethanol affects nervous system function at a number of levels, including endogenous activity patterns in individual nerve cells (1–3), the release of transmitters and hormones from presynaptic terminals (1, 3–6), and the transduction of signals at postsynaptic receptors (7–9). Many of these actions have been traced to alterations in the properties of particular channel populations (10–14). One of the questions concerning the mechanisms that underlie these actions is whether ethanol acts directly on membrane proteins or whether its effects on these proteins result indirectly from actions on the lipid matrix. If the action is direct, can we identify specific sites within the protein where ethanol acts? Such sites have been suggested in the γ-aminobutyric acid receptor, where mutagenesis of a single amino acid can alter the ethanol sensitivity of the γ-aminobutyric acid receptor/Cl^- channel complex (8).

We have been using the *Xenopus* oocyte expression system to examine the effects of ethanol on potassium channels formed from injected cRNAs. This approach offers a number of advantages. Many different potassium channels have now been expressed in this oocyte system (15–18). Fully functional homo-oligomeric channels can be formed from the introduction of a single species of cRNA into the oocyte (15, 18, 19). Because the cDNAs encoding subunits have been sequenced, we can attempt to correlate the deduced amino acid sequence with ethanol sensitivity. Potassium channels have been classified, based upon extent of sequence homology, into a number of subfamilies (20–22). Members of the same subfamily share >70% sequence homology, whereas members of different subfamilies generally share <40% sequence homology. Thus, we can ask whether particular aspects of channel function and pharmacological properties are common to the members of a subfamily. In addition, the types of potassium channel encoded by the different cRNAs injected exhibit a range of functional properties, such as fast inactivation (I_A-type, i.e., ShA1 and ShB1) versus slow inactivation (delayed rectifier-type, i.e., ShabII, RkShIIIA, and DRK1, as well as I_{SK}, which continually activates even during pulses lasting several minutes). Also differing among clones used are pharmacological characteristics, such as sensitivity to tetraethylammonium ions or 4-aminopyridine. Therefore, we can ask whether sensitivity to ethanol is correlated with these properties. Finally, the cRNAs used in this study have widely divergent phylogenetic origins, making it possible to discern patterns in ethanol action related to this.

Perhaps most important, for obtaining an understanding of the actions of ethanol at the molecular level, is the burgeoning body of data relating particular regions of the primary sequence of the channel protein to particular functions of the channel. For example, a stretch of 83 amino acids in the amino terminus of certain potassium channels appears to comprise a "ball-and-chain" structure important in the fast inactivation of the channel (23, 24). Thus, it may be possible to relate the actions of

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ABBREVIATION: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ethanol on particular channel functions to interactions of the drug with specific regions of the protein. Moreover, the ability to mutate selectively potassium channels allows the testing of proposed relationships between primary sequence and sensitivity to ethanol action.

Because the different channel proteins that we are studying are expressed in the same oocyte membrane, we avoid the complications inherent in more complex systems, where differences in ethanol sensitivity of currents among various cells may be attributed either to dissimilarities in the protein or to differences in the lipid environment of the protein. For example, in previous work, where it was found that fast-inactivating potassium channels in distinct cells of the *Aplysia* nervous system differed in their ethanol sensitivity (12, 25), it was impossible to determine whether the dissimilarities resulted from differences in the channel proteins or from differences in the surrounding lipids. Finally, by examining oocytes in which essentially all of the examined current results from expression of a single species of channel, we avoid the need to isolate electrically the individual channel types, by use of pharmacological blockers, and the possibility that apparent effects on the current under study result from changes in other currents whose degree of block is affected by ethanol.

Experimental Procedures

Materials

Ribonucleotide triphosphates, $m^7G(5)ppp(5)G$, and DNase (RNase-free) were purchased from Pharmacia LKB (Piscataway, NJ). RNasin was obtained from Promega (Madison, WI). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). T3 or T7 RNA polymerase was purchased from either New England Biolabs or GIBCO-BRL (Gaithersburg, MD). HEPES, pyruvic acid (sodium salt), penicillin/streptomycin solution, and theophylline were obtained from Sigma Chemical Co. (St. Louis, MO).

Methods

***In vitro* transcription.** Run-off transcriptions were carried out in a standard transcription buffer (40 mM Tris·HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine HCl, 5 mM NaCl), containing 5 μg of linearized DNA template (see Table 1), 500 μM ribonucleotide triphosphates, 500 μM $m^7G(5)ppp(5)G$, 64 units of RNasin, 2 mM dithiothreitol, and 20 units of RNA polymerase, in a 50-μl volume. After 1 hr at 37°, the templates were digested with DNase I for 30 min at 37°. The cRNAs were extracted in phenol-chloroform, precipitated with ethanol, dried, and then taken up in diethylpyrocarbonate-treated water for injection into *Xenopus laevis* oocytes. The cRNAs thus obtained were checked for homogeneity and size on 1% formaldehyde gels.

Electrophysiology. Adult female *X. laevis* were maintained in artifical pond water at room temperature, on a 12/12-hr light/dark cycle. Dissected stage V and VI oocytes were defolliculated in Ca^{2+}-free saline [82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES (Sigma), pH 7.5], containing 2 mg/ml collagenase (Sigma type IA). Oocytes were maintained in this medium, with continuous shaking at room temperature, for 2–4 hr. After defolliculation, healthy oocytes were selected, and the Ca^{2+}-free medium was replaced with normal Ca^{2+}-containing ND96 medium (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5). This medium was supplemented with 2.5 mM sodium pyruvate, 0.5 mM theophylline, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Oocytes were injected with various cRNAs by using a 10-μl Drummond micropipetter modified for microinjection (Drummond Scientific Co., Broomhall, PA). The micropipette was backfilled with mineral oil and loaded with 2 μl of cRNA in diethylpyrocarbonate-treated distilled water. The cRNA concentrations used are shown in Table 2. After injection of approximately 50 nl of RNA solution, the oocytes were placed in ND96 culture medium, in 24-well plates, and incubated at 18° for at least 2 days before recording. Media were changed every 1–2 days. Oocytes remained viable for up to 7 days after injection.

Oocytes were voltage-clamped in recording medium (normal ND96 culture medium minus sodium pyruvate, theophylline, and antibiotics) at room temperature, using a Dagan 8500 (Dagan Corp., Minneapolis, MN) two-electrode voltage clamp. Shielded electrodes filled with 3 M KCl had resistances of 1–2 MΩ, when measured in ND96. Holding potentials chosen (Table 2) were the same as those cited in the original description or subsequent work on the electrophysiology of each of the clones. They occur at a point on the steady state inactivation curves where minimal inactivation is present (21). Oocytes with resting potentials more depolarized than −50 mV were discarded. Oocytes were continually perfused (1 ml/min) in the recording chamber, which had a volume of approximately 300 μl. At least 15 ml of ethanol were perfused at each concentration before measurements were made. Data were collected and analyzed using PClamp software (Axon Instruments). Capacitative and leak currents were subtracted off-line, using a P/2 protocol (26). Decay time constants were computed by linear regression, using the method of least squares.

Results

The effects of ethanol were tested on members of the Shaker (ShA1, ShB1, RBK1, and RBK2), Shab (ShabII and DRK1), and Shaw (RKShIIIA) potassium channel subfamilies expressed in *Xenopus* oocytes. Also examined were I_{SK} (human

TABLE 1

Molecular biology protocols

After receipt (see Acknowledgments), clones were transformed and propagated in *Escherichia coli* XL-1 (Stratagene) competent cells. Plasmid DNA was isolated using a Qiagen plasmid isolation kit. The potassium channel clones have been isolated from either genomic or cDNA libraries or by expression cloning techniques (see references for details). The species from which these clones were derived are *Drosophila melanogaster* (dros), human (hum), and rat.

Clones	Family	Vector	Linearized	RNA polymerase	Molecular mass[a]	Ref.
					kDa	
ShA1 (dros)	Shaker	SP72	*Hin*dIII	T7	64	48
ShB1 (dros)	Shaker	SP72	*Hin*dIII	T7	74	48
RBK1 (rat)	Shaker	pTZ18U	*Hin*dIII	T7	55	27
RBK2 (rat)	Shaker	pTZ18U	*Hin*dIII	T7	55	49
ShabII (dros)	Shab	pBluescript II KS	*Sac*II	T3	100	9
DRK1 (rat)	Shab	pBluescript II SK	*Not*I	T7	95	28
ΔC318-DRK1	Shab	pBluescript II SK	*Not*I	T7	60	29
RKShIIIA (rat)	Shaw	pBluescript II SK	*Xho*I	T3	62	50
I_{SK} (hum)	Isk	pGEM9Zf(−)[b]	*Not*I	T7	15	51
I_{SK} (rat)	Isk	pUC13	*Pvu*II	T7	15	16

[a] Molecular masses of proteins were deduced from the published cDNA sequences.
[b] A modified pGEMA (Promega) vector modified to include a poly(A) tail.

TABLE 2

Electrophysiological protocols

This table indicates the voltage-clamp protocols used for each of the channels. Although not listed, the protocol used for the deletion mutant ΔC318 was identical to that used for DRK1, except for the pulse duration, which was 0.5 sec; voltage steps were applied in increments of 20 mV, after the initial step, except for ShA1, for which one single step to +40 mV was performed. For determination of decay time constants, in the case of DRK1, ΔC318-DRK1, and ShabII, a single step to +40 mV, with a duration of 10 sec, was used.

	DRK1	ShabII	RKShIIIA	RBK1, RBK2	ShB1, ShA1	I_{sk} rat, I_{sk} human
cRNA injected (ng/50 nl)	10	50	10	10	10	10
Holding potential (mV)	−50	−80	−90	−80	−80	−60
Initial step (mV)	−20	−40	−60	−40	−40	−40
Final step (mV)	+40	+40	+40	+40	+40	+60
Pulse duration (sec)	0.25	0.5	0.25	0.1	0.05	5
Interpulse duration (sec)	10	10	4	5	5	20

and rat) potassium channels, which are structurally very different from the other channel types. Representative current traces obtained under voltage-clamp conditions, in the absence and presence of ethanol, are shown in Fig. 1. The channels examined produced currents that ranged from fast inactivating to noninactivating or very slowly inactivating currents. Uninjected oocytes produced only very small currents, <1% of the amplitude observed in injected oocytes. The influence of ethanol on the following parameters of the potassium current was monitored: 1) peak amplitude, 2) decay time constant, 3) voltage dependency of activation, and 4) recovery from inactivation (for ShA1).

Effect on peak amplitude. Although none of the channel clones tested was highly sensitive to ethanol, there were significant differences in sensitivity among them. The expressed channels were either inhibited by ethanol or insensitive to ethanol at the concentrations tested. None of the channels examined showed an augmentation of current. The effects of various ethanol concentrations on current amplitude are shown in Fig. 2. The most striking finding was that the current amplitudes of DRK1 and RKShIIIA were significantly less sensitive to ethanol than were those of the other channels examined. This insensitivity was most pronounced for DRK1, where there was no significant reduction of current amplitude even in 600 mM ethanol.

Although DRK1 and ShabII belong to the same subfamily, they differ in their ethanol sensitivity. DRK1 contains an especially long carboxyl terminus (>400 residues) (27–29). In order to test whether this might contribute to the ethanol insensitivity of DRK1 current amplitude, we tested a carboxyl-terminal deletion mutant of DRK1, which is missing 318 residues from the carboxyl terminus (ΔC318DRK1). The deletion mutant was intermediate between DRK1 and ShabII in sensitivity of current amplitude to ethanol. Peak current amplitude in 600 mM ethanol was decreased by only 12.6 ± 4.9% in ΔC318DRK1, compared with 33.5 ± 2.7% for ShabII. The slope of the concentration-response relationship for the deletion mutant differed significantly ($p < 0.05$) from that for the parent DRK1.

Effect on decay time constant. To illustrate the compar-

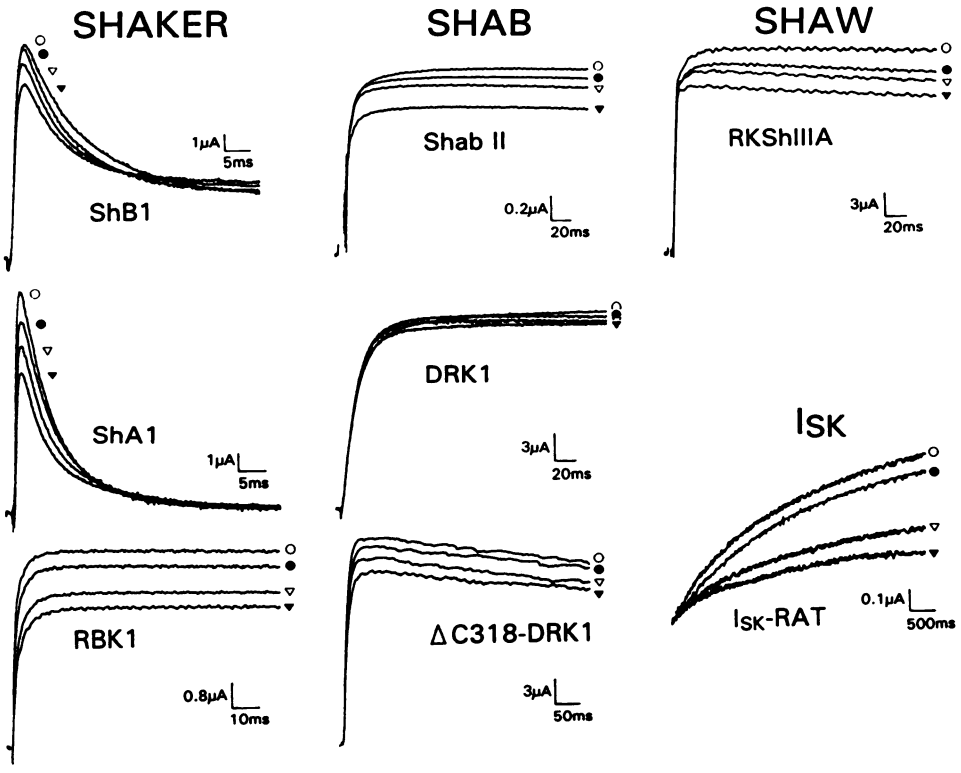

Fig. 1. Currents recorded from *Xenopus* oocytes after injection of cRNA encoding various potassium channels. The oocytes were exposed to increasing concentrations of ethanol. Currents were elicited by steps to +40 mV from holding potentials stated in Table 2. ○, Control; ●, 200 mM ethanol; ▽, 400 mM ethanol; ▼, 600 mM ethanol.

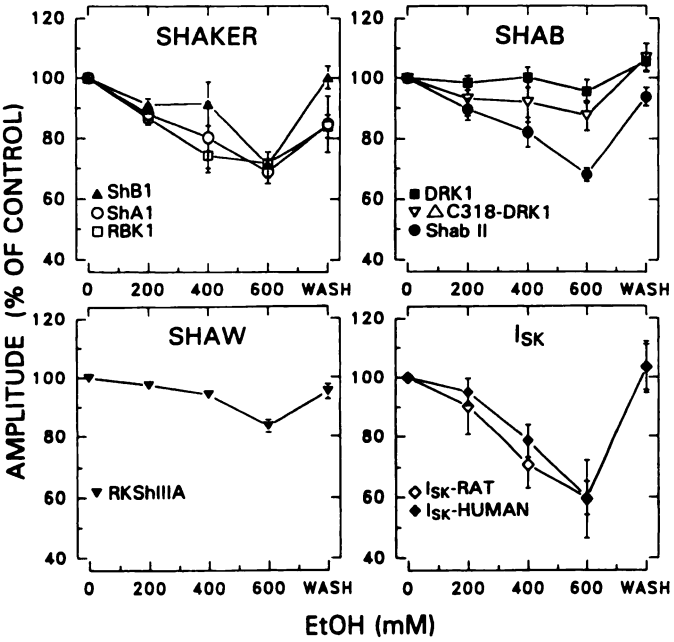

Fig. 2. Plots of mean percentage of change in peak amplitude of potassium current as a function of ethanol (*EtOH*) concentration. Data were averaged from all experiments and show results for a command step to +40 mV. Number of oocytes tested for each data point ranged from 5 to 16. Data for RBK2 (not shown) were similar to those obtained for RBK1. Absolute control values (μA) were as follows: ShB1, 10.9 ± 1.5; ShA1, 6.7 ± 0.8; RBK1, 20.9 ± 3.0; DRK1, 18.8 ± 3.2; ΔC318DRK1, 19.7 ± 5.2; ShabII, 2.3 ± 0.4; RKShIIIA, 4.5 ± 1.2; I_{SK} rat, 0.9 ± 0.2; and I_{SK} human, 1.0 ± 0.1. Ethanol concentrations at which the data for each of the clones became significantly different ($p < 0.05$) from control values were ShA1, ShB1, RBK1, ShabII, and RKShIIIA, 200 mM; I_{SK} human and I_{SK} rat, 400 mM; and ΔC318DRK1, 600 mM. DRK1 was not significantly different.

ative effects of ethanol on the decay kinetics of potassium currents produced from each of the channel clones, we have scaled and superimposed current traces, obtained in the presence of various ethanol concentrations, to the same peak values (Fig. 3). As in the case of current amplitude, the sensitivity to ethanol of decay kinetics differed among clones (Fig. 4). Interestingly, these differences often did not parallel those seen for current amplitude. For example, whereas the amplitude of current produced from DRK1 was insensitive to ethanol, the decay kinetics of the current produced from this clone were among the more sensitive of those sampled. The deletion mutant ΔC318DRK1 showed slightly faster decay kinetics than the parent DRK1 channel (3.6 ± 0.4 versus 5.0 ± 0.5 sec) but did not differ from DRK1 in the sensitivity to ethanol of the decay kinetics, in contrast to the results obtained for current amplitude. As was the case for the ethanol sensitivity of current amplitude, shared membership in a subfamily did not ensure that ethanol would have similar effects on decay kinetics. For example, comparison of decay time constants in 600 mM ethanol showed a statistically significant ($p < 0.05$) difference between ShabII and DRK1, even though both are members of the Shab subfamily. One of the four oocytes expressing RkShIIIA (traces shown in Fig. 3) showed the induction of a fast decay phase (time constant = 0.6 sec and 0.4 sec in 200 and 400 mM ethanol, respectively), in addition to the slower phase. The slow decay phase was relatively insensitive to ethanol in all cases.

Effect on the voltage dependency of activation. Representative current-voltage and conductance-voltage relationships for activation of a number of the potassium channels examined in the absence and presence of ethanol are shown in Fig. 5. The voltage dependency of activation was unaffected, even at 600 mM ethanol concentrations, in all of the channels examined.

Effect on recovery from inactivation. ShA1 differs from other potassium channel clones in that an especially long recovery time is required, after an inactivating voltage pulse, before the channel population can be fully activated by a subsequent depolarization (30). We used a double-pulse protocol, with varying times between a conditioning pulse and the test pulse, to examine whether the time required for recovery

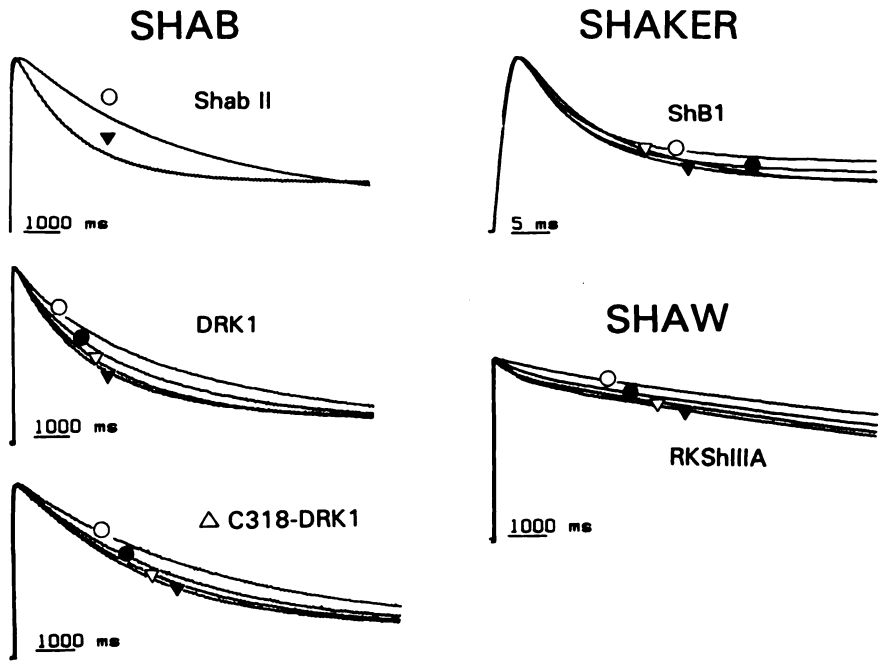

Fig. 3. Effects of ethanol on potassium current decay kinetics. Command steps were to +40 mV from holding potentials stated in Table 2. Traces obtained in ethanol were scaled to control values, to facilitate the comparison of decay kinetics. ○, Control; ●, 200 mM ethanol; ▽, 400 mM ethanol; ▼, 600 mM ethanol.

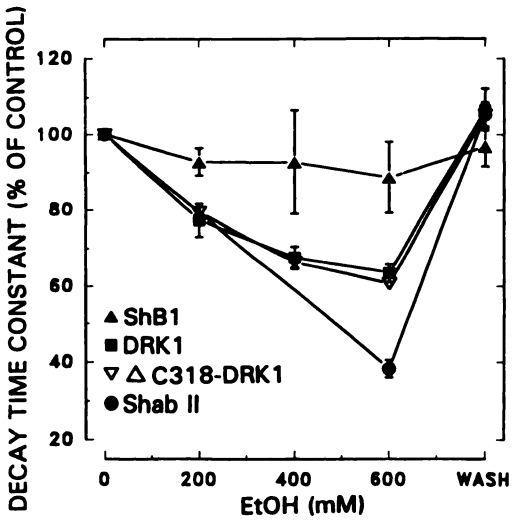

Fig. 4. Plots of the mean percentage of change in the decay time constants of potassium currents at various ethanol (*EtOH*) concentrations. Data were averaged from all experiments and show results for a command step to +40 mV. Number of oocytes tested for each data point ranged from 5 to 13. Time constants were computed for segments of traces between 4 and 45 msec after the voltage step for ShB1 and between 0.8 and 9.0 sec after the beginning of the voltage step for others shown. Absolute values (msec) at control were as follows: ShB1, 9.4 ± 0.9; DRK1, 4964 ± 447; ΔC318DRK1, 3638 ± 384; and ShabII, 3883 ± 175. Ethanol concentrations at which the data for each of the clones became significantly different ($p < 0.05$) from control values were DRK1 and ΔC318DRK1, 200 mM; and ShabII, 600 mM (not tested at lower concentrations). ShB1 was not significantly different.

from inactivation was affected by ethanol. Exposure to concentrations of ethanol as high as 600 mM did not affect the time course of recovery from inactivation of ShA1 (data not shown).

Effect of longer chain alcohols. In a limited number of experiments, hexanol was tested for its effect on peak amplitude of DRK1. As previously shown, the current amplitude of DRK1 was particularly insensitive to ethanol. We chose bath concentrations of hexanol that were likely to produce membrane concentrations comparable to those produced by the ethanol concentrations used in this study (31). The peak amplitude of DRK1 potassium current was decreased to 45.7 ± 4.9% ($n = 3$) of control values in the presence of 6 mM hexanol (Fig. 6). Thus, the insensitivity of DRK1 current amplitude to ethanol is not generalized to all alkanols.

Discussion

It is becoming apparent that potassium channel proteins are composed of discrete regions, which can be assigned specific roles in the functioning of the channel. For example, considerable evidence has accumulated that a ball-and-chain mechanism involving the amino terminus of the polypeptide (24, 32) controls relatively fast (milliseconds) inactivation of the channel. Similarly, the S4 transmembrane region is important for the voltage-dependent gating of the channel (15, 33), and the S5-S6 linker region plays an important role in forming the channel pore and determining the ion specificity (34, 35) of the channel. Our data suggest that ethanol may act differentially on these discrete regions of the channel protein, rather than by perturbing the "global" structure of the protein.

This interpretation is supported by the finding that the effects of ethanol on current amplitude were independent of effects on current kinetics. That is, the channels with the most sensitive peak currents were often among the least sensitive clones with respect to kinetic parameters. Some aspects of channel function were remarkably insensitive to ethanol, suggesting that the corresponding parts of the protein were unaffected by even high concentrations of ethanol. None of the clones showed a shift in the voltage dependency of potassium current in the presence of ethanol. This is similar to results previously reported in *Aplysia* (12) and suggests that the S4 transmembrane region is resistant to ethanol action. The decay of ShA1 and ShB1 currents was not significantly altered by ethanol. Because both of these channels contain the amino terminus ball-and-chain and show fast inactivation, the ball-and-chain is unlikely to be a target for ethanol at the concentrations used here. In *Aplysia*, the relatively fast decay of I_A was retarded in some cells and unaffected in other cells (12), suggesting the possibility that multiple mechanisms for fast inactivation, with differing ethanol sensitivities, may exist. The sensitivity of DRK1 to alkanols shows some specificity, inasmuch as hexanol, at bath concentrations that are predicted to produce membrane concentrations similar to those attained with ethanol, decreased potassium current amplitude in DRK1, which was insensitive to ethanol. Other clones or other chain length alcohols were not tested.

Deletion of 318 amino acids from the carboxyl terminus of DRK1 increased the ethanol sensitivity of current amplitude, suggesting that these residues offer some protection from ethanol perturbation of the channel. Previous workers have

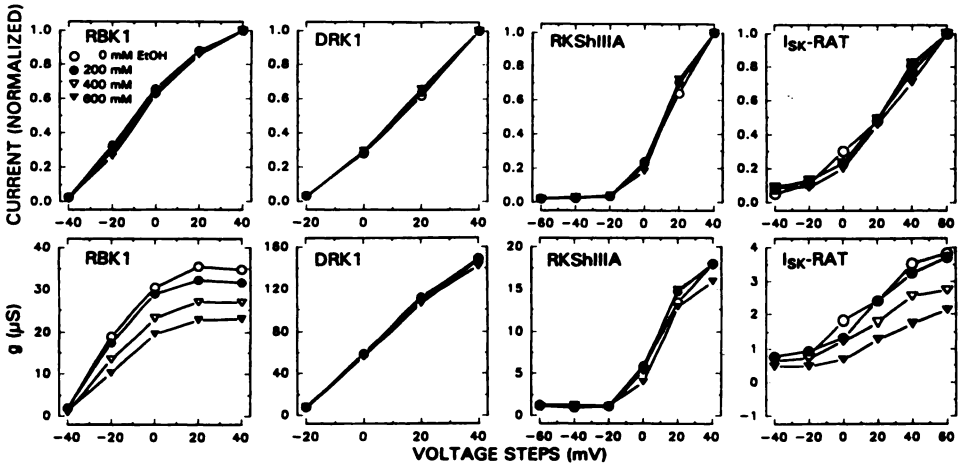

Fig. 5. *Upper*, current-voltage relationships for different potassium channel clones, in the absence and presence of ethanol (*EtOH*). Peak amplitudes obtained at less depolarized step potentials were normalized to the values obtained at the +40 mV command step. *Lower*, conductance-voltage relationships determined for the same data set as represented in *upper*. Conductance was determined as $g = I/\Delta E$. The data indicate that ethanol does not shift the voltage dependency of activation.

TABLE 3

Comparison of amino acid sequences in the pore-forming region of potassium channels

Bold letters indicate amino acid residues that are different in DRK1, compared with the other potassium channels.

K^+ channels	Pore-forming region		Amino acid numbering
DRK1	EKDEDD	TKFKSIPASFWWATITMTTVGYGDIYPKTLLGKI	349–388
ShabII	EKDEKD	TKFVSIPEAFWWAGITMTTVGIRDICPTTALGKV	599–638
ShA1	EAGSEN	SFFKSIPDAFWWAVVTMTTVGYGDMTPVGFWGKI	418–457
ShB1	EAGSEN	SFFKSIPDAFWWAVVTMTTVGYGDMTPVGVWGKI	418–457
RBK1	EAEEAE	SHFSSIPDAFWWAVVSMTTVGYGDMYPVTIGGKI	348–387
RBK2	EADERD	SQFPSIPDAFWWAVVSMTTVGYGDMVPTTIGGKI	348–387
RKShIIIA	ERVGAQPNDPSASEHTQFKNIPIGFWWAVVTMTTLGYGDMYPQTWSGML		404–451

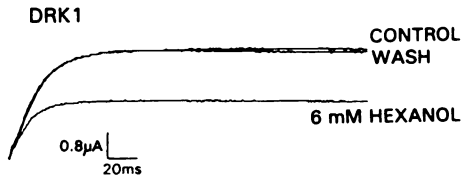

Fig. 6. Effect of 6 mM hexanol on DRK1 potassium current. Traces were elicited by a command step to +40 mV from a holding potential of −50 mV, before, during, and after exposure to hexanol.

suggested that ethanol, especially at high concentrations, exerts its effects on membrane proteins via a perturbation of membrane lipids (36–40). Because present evidence suggests that the carboxyl terminus resides in the cytoplasmic domain, and not in the membrane (28, 29), the effects of ethanol on channel proteins must not be limited to those portions of the channel traversing the lipid domain of the membrane. Of course, we cannot exclude the possibility that different regions of the protein interact in a manner important for the pharmacology of the channel.

Because the pore region of the channel may play a role in the reduction of current amplitude in the presence of ethanol, we compared the amino acids in the extracellular loop between S5 and S6 to determine which were unique to DRK1, in comparison with other clones that were more sensitive to ethanol. This region of the channel protein has been suggested to contribute to the channel pore by traversing the membrane in a β-sheet configuration (34). The amino acid sequences for the loops connecting S5 and S6 of the potassium channels examined here are aligned in Table 3. There are four residues unique to DRK1 in this region, Ala-362, Ser-363, Thr-368, and Lys-382. If individual residues contribute to the differences in ethanol sensitivity, they might be identified by the analysis of point mutants in which the unique residues in DRK1 have been changed to those present in the more sensitive channels. For example, the position corresponding to Ala-362 is an acidic residue in ShabII, RBK1, RBK2, ShA1, and ShB1. Charybdotoxin and tetraethylammonium binding to mutant ShB1 channel suggest that this residue, Asp-431 in ShB1, lies on the outside surface of the membrane (34, 41, 42).

Conceivably, the carboxyl group represents a site for hydrogen bonding of the ethanol hydroxyl group (43), which interferes directly with the functioning of the channel. Interestingly, RKShIIIA, which, like DRK1, has a neutral residue at this position, exhibits an intermediate current amplitude sensitivity to ethanol.

The concentrations of ethanol used in this study are quite high. However, blood ethanol concentrations in excess of 200 mM have been reported to occur in alcoholics (44). In addition, relatively small changes in potassium currents can lead to relatively large differences in physiological processes, such as transmitter release and signal transduction (45–47). Thus, in addition to providing insights into the manner in which high concentrations of ethanol interact with channel proteins, the results may be relevant to the actions of ethanol on membrane proteins in alcoholism.

Acknowledgments

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