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Ethanol Increases the Activity of Large Conductance, Ca^{2+}-Activated K^+ Channels in Isolated Neurohypophysial Terminals

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SUMMARY

Large conductance, Ca^{2+}-activated K^+ channels are believed to underlie interburst intervals and, thus, contribute to the control of hormone release from neurohypophysial terminals. Because ethanol inhibits the release of vasopressin and oxytocin, we studied its effects on large conductance, Ca^{2+}-activated K^+ channels from these terminals using patch-clamp techniques. Ethanol (10–100 mM) applied to the cytosolic surface of excised, inside-out patches reversibly increases channel activity in a concentration-dependent manner, reaching a plateau at 50–100 mM. This activation is not mediated by freely diffusible cytosolic second messengers or the release of Ca^{2+} from intracellular stores. Rather, it likely reflects a direct interaction of ethanol with the channel protein or a closely associated component. Neither the unitary conductance nor the characteristics of the voltage-current relationship are modified by the drug. The increase of channel activity by ethanol results from a modification of channel gating properties: the contribution of long openings to the total time spent in the open state is increased, the average duration of the fast openings is slightly increased, and long closures disappear in the presence of the drug. The activation of large conductance, Ca^{2+}-activated K^+ channels by ethanol, in conjunction with the previously reported inhibition of voltage-dependent Ca^{2+} channels, can explain the reduced release of vasopressin and oxytocin after ethanol ingestion.

CAK channels provide a link between cellular metabolism and membrane potential: a rise in cytosolic Ca^{2+} leads to an enhancement of K^+ efflux, which hyperpolarizes/repolarizes the cellular membrane. For example, the latter part of the AHP that follows single action potentials and bursts of impulse activity in neurons has been demonstrated to be produced, at least in part, by CAK conductances (1, 2). The efflux of K^+ due to CAK channel activation hyperpolarizes the cellular membrane away from the spike threshold, resulting in inhibition or stabilization of the neuron. Thus, CAK currents have been demonstrated to decrease neuronal excitability and control the frequency and duration of neuronal firing (2–5). This has led some authors to speculate that CAK currents may be involved in the depressant action of EtOH and other sedative/hypnotic drugs on central neurons (6, 7).

The effects of EtOH at relevant clinical concentrations (legal intoxication in the United States is ~20 mM) on CAK responses have been documented in a number of studies. Using intracellular recordings, it has been demonstrated in CA1 cells of rat hippocampus that 20 mM EtOH enhanced the Ca^{2+}-dependent AHP, which is attributed to an increase in K^+ conductance, while having no effect on Ca^{2+}-independent AHP (8). Similar findings were reported for CA3 hippocampal neurons and cerebellar Purkinje cells (9) and dentate granule cells from hippocampus (10). Enhancement of both the repolarization phase of the action potential and its AHP were produced by 5–50 mM EtOH in cells F1 from the right parietal ganglion of *Helix aspersa,* reportedly due to enhancement of a CAK conductance (11). In addition, EtOH (100 and 400 mM) and several general anesthetics have been shown to increase the Ca^{2+}-dependent efflux of Rb^{86} (used as a marker for K^+) from resealed human red blood cells (12, 13), a current that has been shown to bear many similarities to the CAK currents present in neurons (14).

In the present study, we used single-channel and whole-cell current recordings to study the effects of EtOH at relevant clinical concentrations (10–100 mM) on large conductance (BK), CAK channels in neurohypophysial terminals, which represent an actual target for the action of EtOH in the body. K^+ channels play a critical role in the secretion of AVP and OT from the neurohypophysis. The blockade of K^+ channels broadens action potentials and enhances peptide

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ABBREVIATIONS: CAK, Ca^{2+}-activated K^+; AHP, afterhyperpolarization; EtOH, ethanol; BK, large-conductance Ca^{2+}-activated K^+; AVP, vasopressin; OT, oxytocin; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; P_o, probability that a particular BK channel is open.

release (15, 16). BK channels have been characterized in rat neurohypophysial terminals, where they are believed to be involved in the regulation of interburst duration and, thus, peptide release (17). In this preparation, EtOH (10–100 mM) has been previously reported to inhibit both voltage-dependent Ca^{2+} channels (18–20) and AVP release (19). Increase of BK channel activity by EtOH together with inhibition of voltage-dependent Ca^{2+} channels would result in inhibition of neuropeptide release, decrease in circulating levels of AVP, and, ultimately, production of diuresis, all well-documented effects of EtOH intake (for review, see Ref. 21).

The use of single-channel recordings allows us to determine the underlying mechanisms responsible for the increase of CAK currents produced by EtOH. Thus, we can determine whether the enhancement of CAK currents by the drug is due to a change in the number of functional CAK channels present in the cellular membrane, to an increase in the single-channel conductance, to a modification of channel gating properties, or to a combination of these possibilities. CAK currents are dependent on both the cytoplasmic concentration of Ca^{2+} and voltage for their activation and are modulated by various second messenger systems. Complications in the study of this channel can arise from the fact that EtOH has been demonstrated to release Ca^{2+} from intracellular stores (22, 23). However, by using selective blockers (24), we can specifically test whether the activation of BK currents by EtOH is secondary to a release of Ca^{2+} from internal stores. The use of excised, inside-out patches enabled us to control the Ca^{2+} concentration at the cytosolic side of the channel, where the Ca^{2+}-sensitive sites are located (25). Then, we can address the role of freely diffusible cytosolic second messengers (including cytoplasmic Ca^{2+}) versus membrane-bound entities in the activation by EtOH of CAK conductances. Activation of the channel in excised patches narrows the possibilities for putative targets for the action of EtOH to components in, or closely associated with, the membrane patch.

The results of the present study demonstrate a reversible increase of BK channel activity induced by EtOH (10–100 mM), as a consequence of a modification of channel gating properties by the drug, under conditions that rule out freely diffusible cytosolic second messengers as mediators of the activation.[1]

Materials and Methods

Preparation. Neurohypophysial terminals from male CD rats weighing 150–200 g (Charles River, Boston, MA) were freshly isolated as previously described (27). The procedure provides nerve endings free from contamination by postsynaptic or other neuronal structures. The dissociated terminals were allowed to attach to the bottom of a dish coated with poly-L-lysine hydrobromide. The individual terminals, typically 3–8 μm in diameter, were readily identified with a combination of phase and interference (Hoffman-modulation) optics. The identity of the neuroterminals could be confirmed by immunocytochemistry of AVP, OT, and neurophysins (27, 28).

Whole-cell recordings. CAK currents were obtained from the nerve terminals using the patch-clamp technique in the whole-cell configuration (29). Data were acquired and stored using an A/D converter and an IBM-compatible computer. All amplitude values of the outward CAK current were measured with a cursor during the plateau of the current, 90–150 msec after the beginning of the voltage step. Currents were leak-subtracted off-line with a P/2 protocol. Currents were recorded using a patch-clamp amplifier (EPC7, List Electronics, Darmstadt, Germany) at a bandwidth of 3 kHz.

Electrodes were pulled from glass capillaries (Drummond Scientific Co., Broomall, PA). The shank of each electrode was coated with Sylgard (Dow Corning Co., Midland, MI) to reduce capacitance and electrical noise. Immediately before recording, the tip of the electrode was fire-polished on a microforge (Narashige, Kyoto, Japan) to give resistances of 3–5 MΩ when filled with intraterminal-like solution consisting of 110 mM KCl, 20 mM *N*-methyl-D-glucamine-Cl, 0.62 mM $CaCl_2$, 2 mM EGTA, 40 mM HEPES, 2 mM Mg-ATP, and 0.2 mM cAMP, pH 7.2 (~100 nM free Ca^{2+} concentration). Precisely buffered calcium solutions were prepared according to Fabiato (30). For whole-cell recordings, the nerve terminals were bathed in physiological solution consisting of 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 15 mM HEPES, 10 mM glucose, and 7 mM 4-aminopyridine, pH 7.35.

Single-channel recordings. Single-channel recordings were obtained from excised, inside-out and outside-out membrane patches using standard patch-clamp techniques (29). Single-channel currents were recorded and stored as described above for whole-cell currents. Unitary single-channel currents were filtered at 1 kHz, with an eight-pole Bessel filter (model 902LPF, Frequency Devices, Haverhill, MA), and sampled at 5 kHz. Electrodes were made as described above for recording of whole-cell currents, but in this case their tips had resistances (using K^+ as the main ion in the solution filling the electrode) of 6–10 MΩ.

Single-channel recordings were obtained in symmetrical conditions, i.e., the same solution bathed both the extracellular and the cytosolic sides of the membrane patch. The solution consisted of 145 mM K^+ gluconate, 1.99 mM $CaCl_2$, 2 mM EGTA, 15 mM HEPES, and 10 mM glucose, pH 7.4 (~7 μM free Ca^{2+} concentration). After excision from the terminal, the membrane patch was exposed to a stream of bath solution containing the desired concentration of EtOH flowing from a micropipette (1 mm diameter; WPI Inc., New Haven, CT). Bath solution with dextrose isosmotically replacing EtOH was used as the control perfusion. All experiments were carried out at room temperature.

Data acquisition and analysis were performed using pCLAMP software, version 6.0.2 (Axon Instruments, Burlingame, CA). The total average BK current (I_{BK}) flowing through a membrane patch is given by: $I_{BK} = NP_o i$ where N is the number of BK channels present in the patch membrane, P_o is the probability that a particular BK channel is open, and i is the current single channel.

In this study, we explored which of these parameters were affected by EtOH, resulting in an increase of BK currents in neurohypophysial terminals. As a measure of channel activity, we used the product NP_o, which was obtained from all-points amplitude histograms. For construction of these histograms, data were sampled for a period of 21–43 sec. Because a second or third application of EtOH to a patch was not as effective in increasing channel NP_o as the first application, all NP_o values in the presence of EtOH were taken from the first exposure to the drug. Unless otherwise stated, data are expressed as mean ± standard error (with the number of different terminals or number of different membrane patches given in parentheses; in the latter case, each patch was obtained from a different nerve terminal). Least-squares and maximum-likelihood minimization routines were used to fit exponential curves to the distributions of open and closed times. Determination of the minimum number of exponential terms for adequate fit was established using a standard F statistic table.

Chemicals. EtOH (deionized, 100% purity) was purchased from American Bioanalytical (Natick, MA); 4-aminopyridine, ruthenium red, ATP, cAMP, and poly-L-lysine were purchased from Sigma Chemical Co. (St. Louis, MO); and caffeine was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA).

[1] Preliminary data have been presented in abstract form (26).

Results

EtOH increases channel NP_o in excised, inside-out patches. The action of EtOH was studied on a CAK channel subtype that showed features typical of BK channels (17). In excised, inside-out patches, the first exposure of the cytosolic side of the patch to EtOH reversibly increased BK channel activity (i.e., increased NP_o) (Fig. 1). Exposure of the excised patch to a stream of bath solution with dextrose isosmotically replacing EtOH (control perfusion) failed to increase channel activity, which indicates that the activation of BK channels by EtOH is not due to either a flow or an osmotic effect. The fact that EtOH-activation of BK channels was observed in inside-out patches 30 min after the patch membrane was excised from the terminal indicates that this activation is not mediated by freely diffusible cytosolic second messengers. We also tested the possibility that EtOH could release Ca^{2+} from internal stores still attached to the patch membrane after excision, which, in turn, would increase BK activity. Then, we applied either 10 mM caffeine, a blocker of the inositol-1,4,5-triphosphate-activated Ca^{2+} release receptor and activator of the caffeine-activated Ca^{2+} release receptor (24), or 30 μM ruthenium red, a blocker of both the caffeine-activated Ca^{2+} release receptor and mitochondrial Ca^{2+}-buffering systems (24, 32), on the cytosolic side of inside-out patches for at least 5 min and evaluated their effects on BK channel activity. NP_o values were not modified by either caffeine [102.2 ± 2.46% of control values (n = 5)] or ruthenium red [102.7 ± 2.67% of control values (n = 3)]. Furthermore, when 50 mM EtOH was applied with 30 μM ruthenium red, an increase in channel activity was still observed [317.99 ± 112.1 of control values (n = 3)]. In consequence, the increase of BK channel activity by EtOH is not due to an EtOH-induced release of Ca^{2+} from internal stores still attached to the patch membrane after excision. Rather, it likely reflects a direct interaction of the drug with the channel protein or a closely associated component. At all EtOH concentrations tested

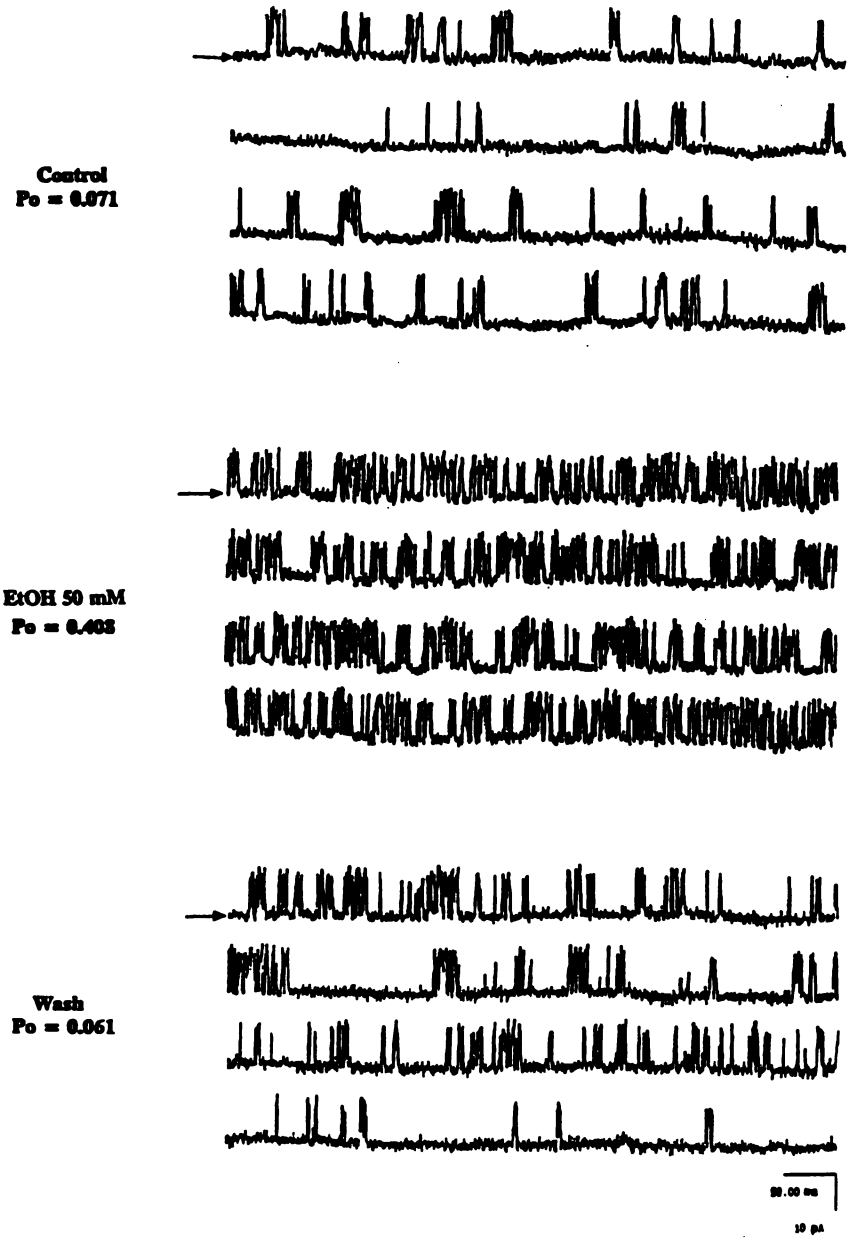

Fig. 1. EtOH (50 mM) reversibly increases BK channel activity in isolated rat neurohypophysial terminals. Representative single-channel recordings obtained from excised, inside-out patches before (*top*), during (*middle*), and 6 min after (*bottom*) exposure of the cytosolic side of the patch to EtOH. *Arrows at the top*, base-line; *upward deflections*, BK channel openings. Four selected traces are shown for each condition; P_o values were calculated from 90–180 sec of recording under each condition. The solution facing the intracellular and extracellular sides of the patch (symmetrical conditions) is described in Materials and Methods. The membrane potential was set to +40 mV.

(10–100 mM), channel activity returned to control NP_o after washout of the drug. The return to base-line values required from 3 to 6 min of drug-free perfusion in all cases, although bath exchange was complete within seconds.

BK activation by EtOH is concentration dependent. The activation of BK channels by EtOH was concentration dependent, from 10 to 50 mM, exhibiting a plateau in the concentration-response curve between 50 and 100 mM (Fig. 2). Maximal activation corresponded to ~450% of NP_o values found in controls. The potentiation observed at the lowest concentration tested (10 mM) corresponded to 150% of control NP_o values, and the EC_{50} obtained from linear interpolation was ~22 mM, a concentration equal to the legal circulating level in the United States. The results obtained for the different concentrations of EtOH expressed as the ratio of the number of positive results (i.e., increase in NP_o) to the number of patches tested is 4:5 for 10 mM, 4:6 for 25 mM, 4:4 for 50 mM, and 4:4 for 100 mM. Despite this qualitative consistency, EtOH-induced activation showed a high quantitative variability (Fig. 2). Variability in the potentiation of EtOH might be due to the observed variability in NP_o values before the exposure to EtOH, at a given membrane potential and cytosolic Ca^{2+} concentration. For example, control values of NP_o measured at 40 mV and cytosolic free Ca^{2+} concentration of ~7 μM ranged from 0.024 to 0.198. This variability in control NP_o values (Fig. 3) might be a function of different states of activation of the channel (probably related to phosphorylation states of the channel protein, which changes over a time scale of minutes) (33, 34), which would cause temporal variability within a single patch recording. In addition, because each patch was obtained from a different nerve terminal, we must also consider interpatch or interterminal variability. In any case, if variability in control NP_o values were to explain the variability in the activation of EtOH, variability in control NP_o values should correlate with the degree of potentiation by EtOH. However, the data showed no correlation (either positive or negative) between these two variables (Fig. 3).

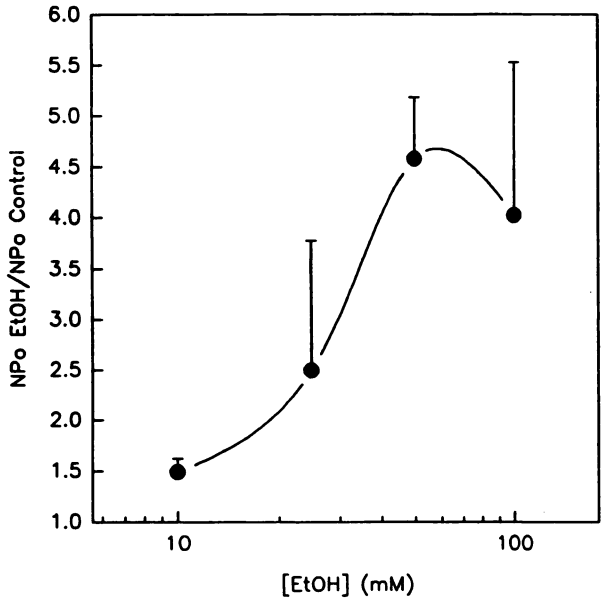

Fig. 2. Increase in BK channel NP_o as a function of EtOH concentration. Results are expressed as the ratio of NP_o values obtained in the presence and absence (before exposition) of the drug, determined in the same patch. Each point of the graph is the mean ± standard error of at least four determinations; each determination was obtained in a different patch, and each patch was excised from a different terminal.

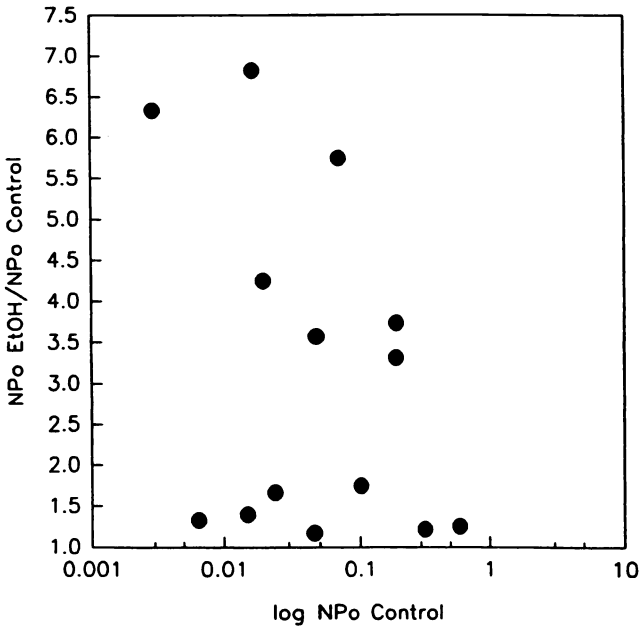

Fig. 3. No correlation is found between the EtOH-induced increase in BK channel activity (expressed as the ratio of NP_o values in the presence and absence of EtOH) and NP_o control values (i.e., before the exposure to EtOH). Each ratio point was plotted against its correspondent pre-EtOH log NP_o value, that is, obtained in the same patch. All points, obtained at the different concentrations of EtOH tested (10–100 mM), were included.

EtOH-induced increase of NP_o does not involve changes in ion conduction properties. BK channels, described in a wide variety of preparations, including rat neurohypophysial terminals (17), are characterized by several key features, which include voltage and Ca^{2+} dependency of channel activity, a large unitary conductance (>150 pS) (25), and a very high selectivity for K^+ (they conduct K^+ ~10-fold more effectively than rubidium) (35). In agreement with these general characteristics, the channels under study increased activity with depolarization of the patch membrane (~10–15 mV/*e*-fold change in NP_o) and/or elevation of the Ca^{2+} concentration (1–10 μM) at the intracellular side of the patch. As shown in Fig. 4, the unitary current/voltage relationship exhibited a reversal (i.e., zero current) potential close to 0 mV in symmetrical 145 mM K^+ gluconate, with no rectification in the range of potentials tested (from −60 to +60 mV in symmetrical zero Mg^{2+}). Under these conditions, the single-channel conductance obtained from slope measurements was 218.3 ± 5.3 pS [r = 0.996 (n = 16)]. As seen in Fig. 5, a representative all-points amplitude histogram of data collected at +40 mV in the presence of 50 mM EtOH (a concentration that produced a significant increase in channel activity, as observed in Figs. 1 and 2) shows that the drug did not change the unitary current amplitude of the channel (mean ± standard deviation, obtained from the gaussian fit): 8.65 ± 2.61 pA in the absence and 8.55 ± 2.25 pA in the presence of EtOH. Furthermore, from the unitary current/voltage relationship (Fig. 4), it can be seen that EtOH at the highest concentration tested (100 mM) neither modified the reversal potential (near 0 mV) nor induced any rectification

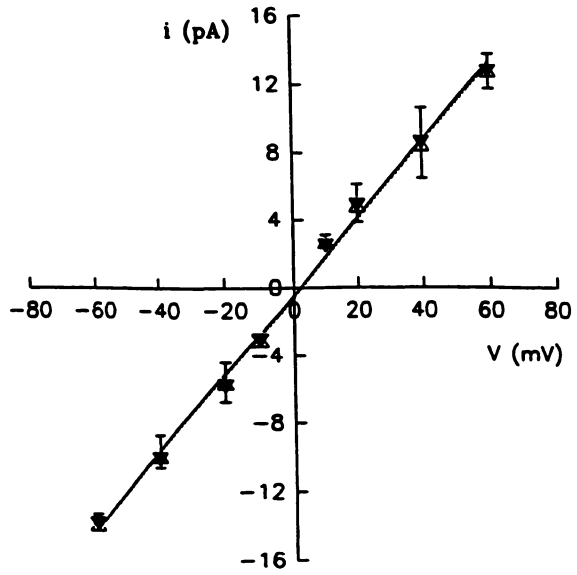

Fig. 4. EtOH (100 mM), the highest concentration tested that increased BK channel activity, does not modify the unitary current/voltage (I-V) relationship of BK channels, measured in inside-out patches. Individual points at each potential were obtained from all-points histograms, and plots were fitted using linear regression. The single-channel conductance, calculated from slope measurements, is 217.7 ± 14.1 pS (r = 0.993) and 218.3 ± 5.3 pS (r = 0.996) in the presence [▲ and *dotted line* (n = 4)] and absence [△ and *solid line* (n = 16)] of EtOH, respectively. In both cases, the reversal potential is near 0 mV, and no rectification in the range of −60 to +60 mV is observed. Data are expressed as mean ± standard error.

or shift in the range of −60 to +60 mV. The single-channel conductance taken from slope measurements was 217.7 ± 14.1 pS [r = 0.993 (n = 4)] and was not significantly different from the value obtained with controls.

The increase in NP_o is due to EtOH-induced changes in channel gating properties. Activation of BK channels by EtOH (i.e., increase in NP_o) can be interpreted, even in multichannel patches (in which N is most probably unknown), as an increase in Po. The use of inside-out (i.e., cell-free) patches makes it very unlikely that EtOH increases NP_o by incorporating new channel proteins into the patch membrane. Moreover, the increase in P_o by EtOH was observed in inside-out patches in which N = 1 (e.g., Fig. 1). The N value was determined by applying a high Ca^{2+} solution (>10 μM) to the cytosolic side of the patch, effectively raising the P_o to ~1. Under this condition, a second level of openings was not observed. Thus, the reversible increase in channel activity by EtOH is most likely due to an increase in P_o. An increase in P_o by EtOH could be explained by an increase in the average duration the channel dwells in the open state (increase in the mean open time), a decrease in the average duration the channel dwells in the closed state (decrease in mean closed time), or both. By studying the distribution of mean open times and mean closed times in the presence and absence of EtOH, we can obtain useful information about the number of conducting and nonconducting states in which the channel dwells and their modification by the drug, since in single-channel analysis it is assumed that the number of terms in the exponential distribution of open and closed times reflect the number of states in the system. For these determinations, we recorded channel events for at least 5 min under each condition (control or EtOH).

The kinetic analysis of BK activity in the nerve terminals shows that openings could be well fitted by two exponentials (Fig. 6A). The analysis also yielded the contribution of each component to the fit (i.e., to the total time spent in the open state), which is given in parentheses. EtOH (50 mM) slightly increased the duration of the fast component of the openings and markedly increased the contribution of longer openings to the total time spent in the open state, from ~50% to 80% (Fig. 6B).

In patches in which N = 1, we could also analyze the distribution of closures and its modification by EtOH. In control medium, the distribution of closed times could be best fitted by three exponentials (Fig. 7A). Again, the contribution of each component to the total time spent in the closed state is given in parentheses. EtOH (50 mM) decreased the average duration of the fast and main contributory component of the closures from 0.506 to 0.399 msec without significantly altering its contribution to the total time spent in the closed state (89.4 versus 87.3%). In addition, EtOH suppressed the longest component of the closures, which accounted for 10% of the distribution in control, such that two exponentials were then sufficient to fit the distribution (Fig. 7B). Collectively, the results shown in Figs. 6 and 7 indicate that BK channel activation by EtOH in neurohypophysial terminals is the consequence of EtOH-induced modifications of channel gating properties. Whether these modifications are produced by drug-induced changes in the voltage and/or Ca^{2+} sensitivity of the channel is currently unknown.

EtOH activates BK currents under physiological conditions. To evaluate activation of the channel by EtOH, we also used physiological solutions on both sides of the membrane. Because the intraterminal concentration of Ca^{2+} is very low (<250 nM when extracellular Ca^{2+} concentration is 2 mM) (32), channel activity would also be correspondingly very low, making it very difficult to collect a sufficient number of events from a single patch. Thus, we decided to evaluate the action of EtOH on whole-cell BK currents.

In this macroscopic current study, 7 mM 4-aminopyridine was present in the bathing medium. This concentration has been reported to fully inhibit the fast, transient K^+ current (the A-current) in these nerve terminals (17). As shown in Fig. 8, 50 mM EtOH was able to increase the average voltage-dependent outward current evoked by stepping the potential to +70 mV from a holding potential of −50 mV. This current, evaluated 90–150 msec after the voltage step, was previously characterized as a BK current (17). With the low intraterminal Ca^{2+} concentration used in our recordings, the average outward current (steady state conditions) evaluated in different terminals (n = 5) ranged from 20 to 50 pA when stepped from −50 mV to +40 mV and from 24 to 98 pA when stepped to +70 mV. EtOH (50 mM) increased this current at all step potentials tested (as percentage of respective control): +40 mV, 130.8 ± 15.4; +50 mV, 140.7 ± 27.8; +60 mV, 140.8 ± 16.3; and +70 mV, 139.7 ± 16.7 (four).

We also found BK channel activation by 50 mM EtOH in excised, outside-out patches (in symmetrical 7 μM free Ca^{2+} concentration). The activation of BK channels when EtOH is applied to either the intracellular (inside-out patches) or the extracellular (outside-out patches and whole-cell recordings) side of the membrane is consistent with the well-known movement of EtOH, a small uncharged amphophil, through lipid bilayers and natural membranes.

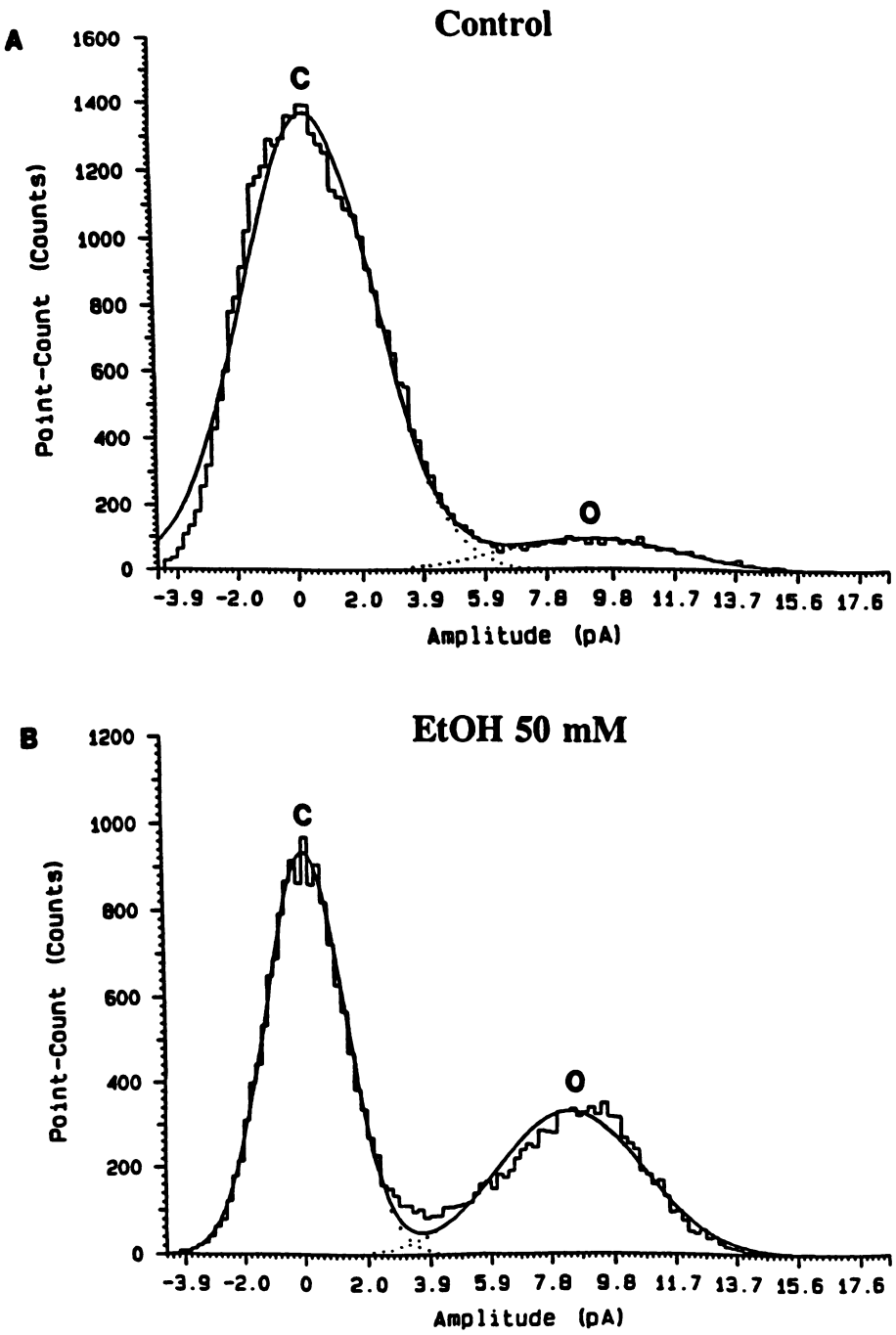

Fig. 5. Effects of EtOH on BK single-channel amplitude. A representative all-points amplitude histogram of the currents recorded from the same terminal in the absence (A) and presence (B) of 50 mM EtOH is shown. *C* and *O* indicate the closed state and the open state, respectively. *Dotted lines*, different components of the fit. Unitary current amplitudes, obtained from gaussian fits, were 8.648 ± 2.605 and 8.551 ± 2.253 pA (mean ± standard deviation) in the absence and presence of EtOH, respectively. Data were sampled for 21 sec (A) and 43 sec (B).

Discussion

In the present report, we demonstrated that EtOH at relevant concentrations (10–100 mM) reversibly increases BK channel activity in neurohypophysial terminals, a system that is an actual target of the action of EtOH in the body (19, 21). This finding provides a probable explanation, at the single-channel level, for the previous observations of an increase in CAK responses in the presence of similar concentrations of the drug (8–13).

The EtOH-activated CAK channels studied here show the typical properties of BK channels, a group defined by their single-channel conductance (>150 pS) and their high selectivity for K^+, as well as characteristic kinetic and pharmacological properties (25, 35). BK channel activity has been postulated to be involved in the regulation of AVP release from the neurohypophysis (17). Activation (i.e., increase in NP_o) of BK channels by EtOH is concentration dependent, showing a plateau between 50 and 100 mM. Interestingly, this plateau occurs at the same EtOH concentrations at which the inhibitory action of EtOH on depolarization-induced AVP release from the terminals also plateaus (19). Furthermore, the EC_{50} value for EtOH-induced BK channel activation (22 mM) is in the same range as that of the IC_{50} value (23.5 mM) for the inhibition by EtOH of voltage-dependent Ca^{2+} channels in this preparation (18, 20). An activation of BK channels, coupled with the already reported inhibition of voltage-dependent Ca^{2+} channels in these nerve terminals, would result in a synergistic inhibition of neu-

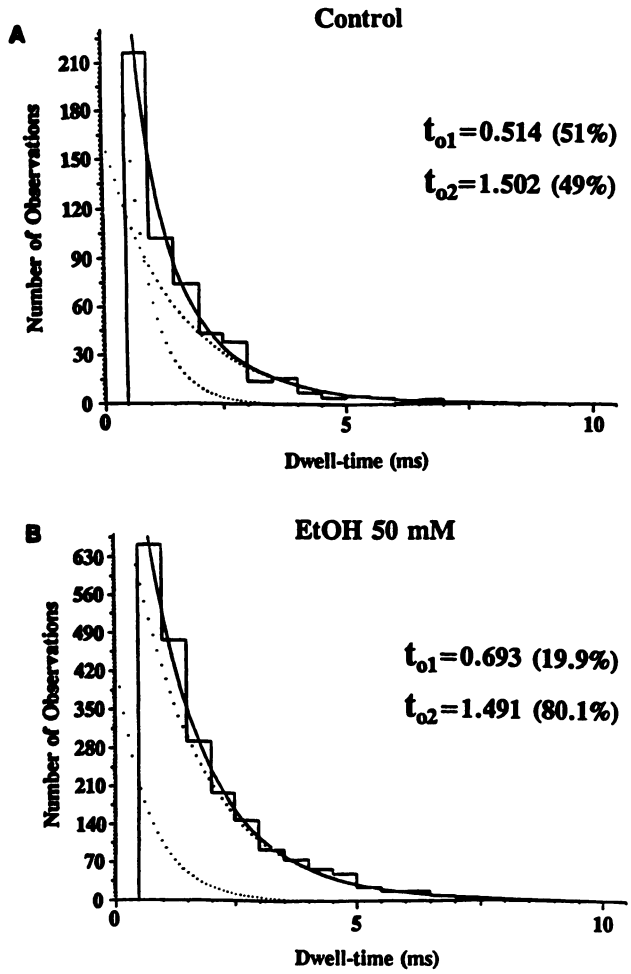

Fig. 6. Open-time histograms in control (A) and 50 mM EtOH (B) obtained with the membrane potential set at +60 mV. In both cases, the distribution of open-times could be well fitted with two exponentials. The different components of the exponential distribution are indicated by dotted lines. A statistical comparison of different fitting models was done using a standard *F* statistic table. *Right*, both the actual time constant values (in msec) and the contribution of each component to the total distribution of openings (as percentages in parentheses) are given. These percentages reflect the relative contribution of each component to the exit from the particular state in which the channel dwells. Number of events was 528 (A) and 2119 (B).

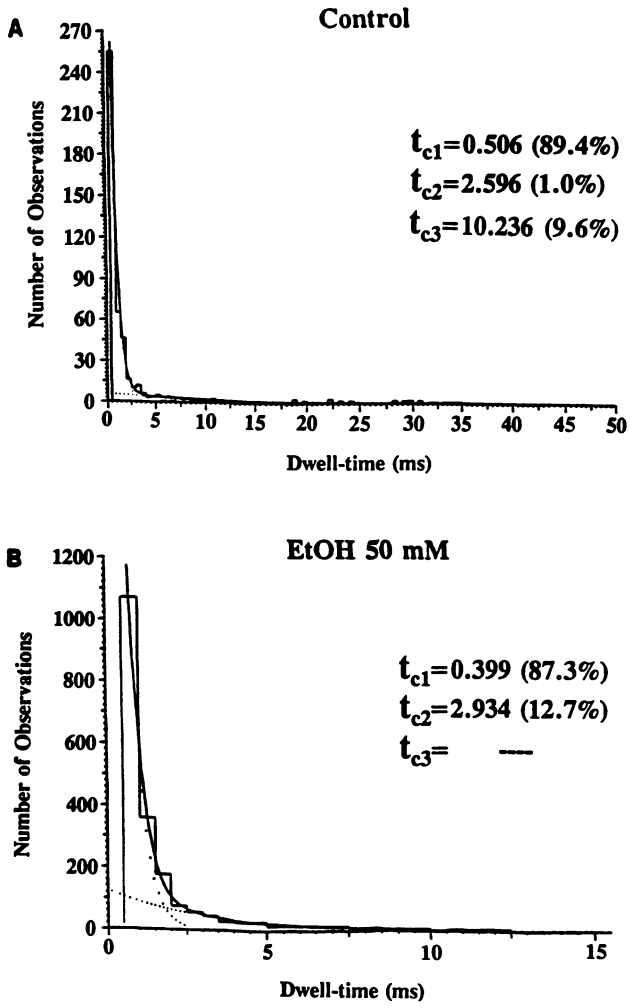

Fig. 7. Closed-time histograms in control (A) and 50 mM EtOH (B) obtained from the same transitions as in Fig. 6. In control, the distribution of closed-times could be best fitted with three exponentials, but in EtOH the longest component was no longer observed, with two exponentials being sufficient to fit the closures. *Dotted lines*, different components of the exponential distribution. A statistical comparison of different fitting models was done using a standard *F* statistic table. Note the different time scale (*x*-axis) in control and EtOH. *Right*, both the actual time constant values (in msec) and the contribution of each component to the total distribution of closures (as percentages in parentheses) are given. For the meaning of these percentages, see Fig. 6 legend and Results. Number of events was 527 (A) and 2,131 (B).

ropeptide release, a decrease in circulating levels of AVP, and, ultimately, production of diuresis, all well-documented effects of EtOH.

The resolution of single-channel recordings allows elucidation of a number of aspects of EtOH-mediated potentiation of CAK channel activity. Thus, we can ask whether an increase of CAK currents by EtOH is due to (i) an increase in the unitary channel conductance, (ii) a change in the number of functional CAK channels present in the cellular membrane, (iii) a modification of channel gating properties, or (iv) a combination of these possibilities. We show here that even the highest concentration of EtOH tested (100 mM) does not increase the unitary channel conductance (218 pS). Despite their large conductance, BK channels are characterized by a very high selectivity for K^+. They conduct K^+ ~10-fold more effectively than Rb^+ and are practically impermeant to Na^+ and Cs^+ (35). EtOH failed to change the reversal potential or induce any rectification in the current/voltage relationship. Together, these findings indicate that ion conduction is unaffected by the drug and suggest that EtOH most likely does not interact with the pore-forming region of the channel.

An increase in NP_o of BK channels by EtOH can be interpreted, even in multichannel patches (in which N is most probably unknown), as an increase in P_o. The possibility that EtOH causes an increase in NP_o by introducing more channels into the patch is unlikely due to the fast and reversible nature of the activation. Also, in excised, inside-out patches, the source of these additional channels would have to be the limited area of membrane under the rim of the pipette, which also makes this possibility unlikely. Finally, and most important, we determined an EtOH-induced increase in P_o in patches in which $N = 1$. Thus, even if an EtOH-induced increase in N occurred, it could not, by itself, account for the increase in channel NP_o induced by the drug. In other words,

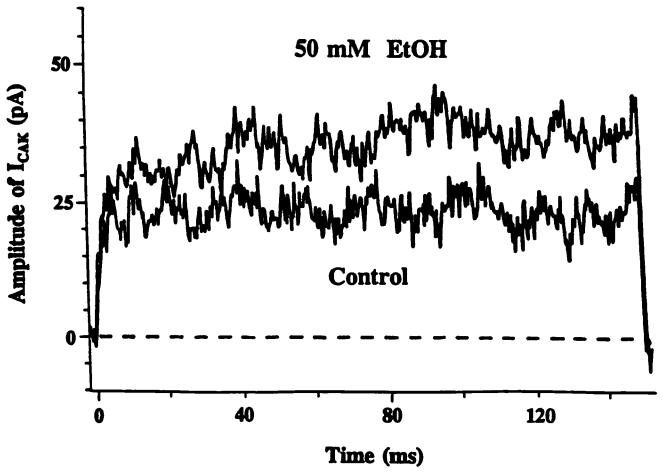

Fig. 8. Activation of BK currents by acute exposure to EtOH under physiological conditions. Representative current traces in the presence (*top*) and absence (*bottom*) of 50 mM EtOH were recorded from the same nerve terminal in the whole-cell configuration. Traces were digitally filtered at a bandwidth of 1 kHz. Fluctuations in the current level correspond to channel openings and closures. Currents were recorded with 100 nM free Ca^{2+}, 200 μM cAMP, and 2 mM Mg-ATP in the pipette and 7 mM 4-aminopyridine in the bath. In the example shown, the sustained outward currents were elicited from V_H of −50 mV and stepped to +70 mV. Typically, these sustained outward currents, previously characterized as BK currents (29), were evoked by stepping the voltage from V_H of −50 mV to a series of depolarizing pulses in the same terminal and evaluated 90–150 ms after the voltage step. Activation by 50 mM EtOH of the average sustained current (expressed as percentage of control values) was consistently observed at all potentials tested: +40 mV, 130.8 ± 15.4; +50 mV, 140.7 ± 27.8; +60 mV: 140.8 ± 16.3; and +70 mV, 139.7 ± 16.7 (n = 4).

the reversible increase of channel activity by EtOH is mainly, if not totally, due to an increase in the channel open probability rather than an increase in the number of conducting channels.

Our results indicate that an increase in P_o by EtOH results from drug-induced modifications of channel gating properties. The EtOH-induced modifications noted, such as the suppression of the longest component of the closures, the marked increase in the contribution of longer openings to the total time spent in the open state, and the small increase in the duration of fast openings, can account for the increase in BK channel activity produced by the drug.

The finding that activation of BK channels by EtOH was observed in the inside-out configuration, in which the concentration of Ca^{2+} to which the cytosolic side of the channel (where the Ca^{2+}-sensitive sites are located) (25) is exposed maintained constant, makes it very unlikely that channel activation is due to a change in the intracellular concentration of Ca^{2+}. Furthermore, the participation of an EtOH-induced release of Ca^{2+} from putative stores (e.g., inositol-1,4,5-triphosphate-activated Ca^{2+} release receptor and caffeine-activated Ca^{2+} release receptor systems) associated with the excised patch could be ruled out because BK channel activity was unmodified by caffeine or ruthenium red and EtOH was still able to activate the channel. We did not use blockers of intracellular release in the whole-cell current recordings, and it is conceivable that intracellular release could contribute to the augmentation of activity by EtOH in this case. EtOH has been reported to increase cytosolic Ca^{2+} through a release of Ca^{2+} from intracellular stores in a number of preparations (22, 23). However, recent findings obtained using fura-2 determination of cytosolic Ca^{2+} demonstrated that caffeine and ryanodine have no effect on Ca^{2+} buffering in intact neurohypophysial terminals (32).

In addition, activation in excised (i.e., terminal-free) patches rules out any action of EtOH on other freely diffusible cytosolic second messenger system(s). The activation of BK channels by EtOH observed at positive membrane potentials in symmetrical Ca^{2+} concentration also rules out the possibility that activation is secondary to a drug-induced influx of Ca^{2+} through cation channels or nonspecific leak in the patch. Thus, the conditions used in this study narrow the possibilities for targets of the action of EtOH to entities located in, or tightly associated with, the membrane, such as the channel protein itself; other proteins embedded in the bilayer, such as G proteins, which have been shown to modulate BK channel activity in other preparations (36, 37); lipids, reported to regulate BK channel activity in vascular smooth muscle (38, 39); or any interface (lipid/protein, water/lipid, and so on) in the membrane at which a small uncharged amphophil, such as EtOH, could act.

The activation of BK channels both in inside-out patches, where the drug is applied onto the intracellular side of the membrane, and in whole-cell currents and outside-out patches, where the drug is applied onto the extracellular side, is consistent with the well-known movement of EtOH, a small uncharged amphophil, through natural membranes (40). The movement of EtOH across the membrane does not allow us to determine whether the site(s) for the action of EtOH is located in (or closer to) a particular leaflet of the membrane. Interestingly, although activation of the channels by EtOH occurs faster than the temporal resolution of our perfusion system (~1 sec), recovery of BK channel activity to control values requires several minutes of washout, raising the possibility that membrane-bound mediators between the channel protein and EtOH could be involved in the action of EtOH.

BK channel activation by EtOH in the nerve terminals was completely reversible. This reversibility argues against the possibility that a nonspecific action of the drug on the membrane (i.e., a generalized increase in ion leak) could explain the increase in BK current. This view is supported by the fact that the activity of other conductances in the same preparation are unmodified by the drug: 50 mM EtOH failed to modify the activity of stretch-activated cation channels,[2] and the transient K^+ current (known as I_A) is not affected by EtOH at concentrations as high as 100 mM (19). This lack of effect on I_A also indicates that the activation by EtOH of BK channels cannot be seen as a generalized activation by the drug of K^+ conductances in the nerve terminals but, most likely, represents a selective activation of the BK channel population. EtOH (10–100 mM) has been previously reported to inhibit the activity of both L- and N_t-type Ca^{2+} channels (18, 19). Despite showing qualitatively opposite effects (i.e., activation versus inhibition), the actions of EtOH on BK channels and Ca^{2+} channels show many similarities: the drug acts on both at comparable concentrations, it does not modify their unitary conductances, and the final effect results from a modification of channel gating properties (20). However, although L-type Ca^{2+} inhibition by EtOH was not reversed after removal of the drug (18), BK channel activa-

[2] A. M. Dopico, J. R. Lemos, and S. N. Treistman, unpublished observations.

tion was completely reversible within 3–6 min of drug-free perfusion. This difference in recovery from the action of EtOH led us to consider the possibility that different molecular mechanisms might mediate the actions of EtOH on these conductances in the nerve terminals.

In conclusion, we demonstrated the reversible activation of BK channels in rat neurohypophysial terminals by relevant concentrations of EtOH. The increase in channel activity occurs as a consequence of a modification of channel gating properties by the drug and results from an interaction of EtOH with the channel protein or some closely associated entity located in the patch membrane. The activation of BK channels by EtOH, in conjunction with the previously reported inhibition of voltage-dependent Ca^{2+}-channels in the neurohypophysial terminals by this drug, can explain the reduced release of AVP and OT after EtOH ingestion.

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