Ethanol directly modulates gating of a dihydropyridine-sensitive Ca2+ channel in neurohypophysial terminals

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Ingestion of ethanol results in a decreased level of plasma vasopressin, which appears to be caused by inhibition of arginine vasopressin (AVP) release from the neurohypophysis. Activation of membrane voltage-gated Ca\(^{2+}\) channels plays an important role in triggering this neurohormone release. In this article, single-channel recordings are used to demonstrate that ethanol, at concentrations constituting legal intoxication, inhibits dihydropyridine-sensitive "L-type" Ca\(^{2+}\) channels in isolated nerve terminals of the rat neurohypophysis. Ethanol reduced the channel open probability in a concentration-dependent manner. To allow finer resolution of channel openings and to better characterize the mechanisms of ethanol action, Bay K 8644 was used to prolong the openings of L-type Ca\(^{2+}\) channels. In the presence of this dihydropyridine (DHP), the reduction of the channel open probability by concentrations of ethanol of 25 mM or higher could be determined to be due primarily, although not completely, to a shortening of the open duration of this L-channel. Channel conductance was unaffected by ethanol, even at high concentrations. These results are consistent with previous macroscopic data indicating that calcium channels in these peptidergic terminals are targets for ethanol action, and indicate that ethanol acts directly on the gating characteristics of the L-type channel. Furthermore, examination of open and closed state transitions, as well as Hill plot analysis, suggests that ethanol's effects on gating are consistent with the interaction of a single drug molecule with a single target site, possibly the L-channel itself.

**Key words: ethanol, calcium channels, neurohypophysis, vasopressin, drug action, dihydropyridines**

Ingestion of ethanol leads to a pronounced reduction in plasma vasopressin levels and an associated increase in diuresis in humans (Nicholson and Taylor, 1938; Eggleton, 1942; Van Dyke and Ames, 1951; Cobo and Quintero, 1969; Eisenhofer and Johnson, 1982), rats (Bisset and Walker, 1957), and dogs (Milerschoen and Riggs, 1969). One drug target underlying this response to ethanol has been suggested to be the neurohypophysial terminals, which originate from cell bodies in the supraoptic and paraventricular nuclei of the hypothalamus. Isolated nerve terminals were prepared from male CD rats weighing 155-195 gm (Charles

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Figure 1. Neurohypophysial terminal unitary L-type Ca\(^{2+}\) channel currents, elicited by repeated steps to +10 mV [holding potential (V\(_h\)) = -50 mV]. A-D. Representative single-channel current traces recorded from a nerve terminal before (left) and after (right) exposure to indicated concentrations of ethanol.

3 pA

20 ms

River, Boston, MA). Details of the preparation have been described previously (Cazalis et al., 1985; Lemos and Nowycky, 1989; Wang et al., 1991a). Briefly, the terminals were dissociated by homogenizing the posterior lobe of the pituitary in a solution containing 270 mM sucrose, 10 mM HEPES, and 10 μM EGTA. The dissociated terminals were attached to the bottom of a dish coated with poly-L-lysine hydrobromide (molecular weight, 15,000–30,000; Sigma, St. Louis, MO). The isolated terminals were viewed under Hoffman modulation-contrast optics. We have previously confirmed, by dot blotting the contents from individual terminals for immunoreactive AVP or oxytocin, that the structures being recorded from were actually neuropeptide-containing terminals (Wang et al., 1991a, 1993a).

Patch-clamp recording. Currents through single Ca\(^{2+}\) channels, with Ba\(^{2+}\) as the charge carrier, were recorded using the “terminal-attached” mode. Patch-clamp currents were amplified with an EPC-7 patch-clamp amplifier (List-Electronic, Germany). Unitary single-channel currents were filtered at 1 kHz, with an 8-pole Bessel filter (mode-902LPF, Frequency Devices, Inc., Haverhill, MA), and sampled at 10 kHz. The interpulse interval was 4 sec. Electrodes were pulled from glass capillaries (Drummond Sci., Broomall, PA). The shank of each electrode was coated with Sylgard (Dow Corning, Midland, MI) to reduce capacitance and electrical noise. Prior to recordings, the tip of the electrode was fire polished on a microforge (Narashige, Kyoto, Japan) to give resistances of 10-12 MΩ. “Terminal-attached” configurations with seal resistances of 50–100 GΩ were produced by gentle mouth suction applied to the back of the electrode. All current traces were corrected for leak currents and capacitative transients. All the recordings were performed on isolated nerve terminals 5–8 μm in diameter, at room temperature (20–25°C).

In a number of patches, all single-channel currents disappeared within the first minute of exposure to even the lowest concentrations of ethanol used in this study, and activity could not be recovered. This case was easily differentiated from the more common case, in which there was only a small inhibition in 10 mM ethanol and further inhibition as the ethanol concentration was increased. Since this rapid loss of channel activity has not been seen in other situations in which the terminal was moved between the different perfusion pipettes, it may represent a category of ethanol response. However, we did not continue the experiment...
if all activity was immediately abolished at low ethanol concentrations, and we cannot report on the mechanisms underlying this particular phenomenon.

**Chemicals and solutions.** The patch pipette solution contained in (mm) BaCl<sub>2</sub>, 110; HEPES, 10; tetraethylammonium chloride (TEA-Cl), 10; pH 7.2. After a seal between the patch pipette and the terminal membrane was obtained in low Ca<sup>2+</sup> Locke solution (in mm: NaCl, 145; HEPES, 10; glucose, 10; KCl, 5; EGTA, 2; CaCl<sub>2</sub>, 1.9; MgCl<sub>2</sub>, 1; pH 7.2), the patched terminal was lifted into a stream of high K<sup>+</sup> external solution from a micropipette (1 mm diameter; World Precision Instruments, Inc., New Haven, CT). The high K<sup>+</sup> external solution contained in (mm) K<sup>-</sup>-gluconate, 145; HEPES, 10; glucose, 10; EGTA, 5; pH 7.3. This high K<sup>+</sup> external solution was used to zero the terminal membrane potential (Fox et al., 1987). Another stream of high K<sup>+</sup> external solution together with ethanol (final concentrations of 10, 25, 50, and 100 mM) flowed from a different micropipette. We have previously shown that the effects of ethanol on calcium channels do not result from osmotic changes, at these concentrations (Wang et al., 1991a,b). Bay K 8644 (Research Biochemicals Incorporated, Natick, MA) was dissolved in ethanol, and then diluted in the pipette solution (final ethanol concentration = 3 mM).

**Data analysis.** The total current observed macroscopically, (I), may be represented as (I) = N x P<sub>o</sub> x i, where N represents the number of conducting channels, P<sub>o</sub> represents the probability of a channel being open, and i represents the current carried through an individual channel. This study explored which of these parameters are affected by ethanol, resulting in the reduction of calcium current and AVP release. Single-channel open times were analyzed with PCLAMP software (Axon Instruments Inc., Burlingame, CA) with transitions detected by the 50% threshold method. Channel activity was shown as the mean channel open probability, that is, “NP<sub>o</sub>,” where N was the number of channels in the patch, and P<sub>o</sub> was the percentage of channel open time over the entire voltage pulse. Histogram drawings of data were accomplished using SIGMAPLOT scientific graphing software (Jandel Scientific, Corte Madera, CA). Most data were expressed as the mean ± SEM (n = number of terminals). The least squares minimization routine was used to fit single- and double-exponential curves to channel open and closed duration distributions. The number of openings used for analysis during control recordings and ethanol exposure ranged from 900 to 3000.

**Results**

**Effects of ethanol on L-type Ca<sup>2+</sup> channel currents**

Terminal-attached recordings were performed with 110 mM Ba<sup>2+</sup> as the charge carrier in the patch electrode. L-type Ca<sup>2+</sup> channel currents in the isolated neurohypophysial terminals have been previously characterized based on their voltage sensitivities, kinetic properties, and responsiveness to DHPs using both whole-terminal macroscopic current and single-channel current recordings (Lemos and Nowycky, 1989; Wang et al., 1993b). L-type channels, selectively evoked by holding potentials more depolarized than −50 mV, had a slope conductance of 25 pS (Wang et al., 1993b). This channel opens intermittently throughout a 120 msec voltage pulse (Fig. 1, left), and is inhibited in a concentration-dependent manner when ethanol is applied in the bath (Fig. 1, right). The channel mean open probability was reduced in the presence of ethanol within 60–80 sec of exposure, and this reduction was concentration dependent (Fig. 2). Currents did not return to control values within the time in which it was possible to maintain stable recordings (30–60 min), even after replacement of the ethanol-containing solution with control medium.

**Effects of ethanol on the L-type Ca<sup>2+</sup> channel currents in the presence of Bay K 8644**

Because of the low frequency of Ca<sup>2+</sup> channel openings and their very short duration (~1 msec), it was difficult to quantitate further ethanol’s actions on Ca<sup>2+</sup> channel currents. Therefore, Bay K 8644, a DHP calcium channel agonist, was utilized. Bay K 8644 activates L-type Ca<sup>2+</sup> channels in these isolated nerve terminals by prolonging channel openings without affecting the slope conductance (Lemos and Nowycky, 1989; Wang et al., 1993b). Bay K 8644 (5 μM) significantly prolonged the channel open time, evident when the traces in Figure 3 (left) were compared with the traces obtained from the nontreated terminals (Fig. 1, left). Furthermore, Figure 3 shows that single-channel Ca<sup>2+</sup> currents, in the presence of Bay K 8644, were inhibited by ethanol. The inhibitory effects of ethanol were qualitatively similar with or without Bay K 8644. However, Bay K–treated terminals were more sensitive to ethanol, particularly at low concentrations. In the presence of Bay K 8644, 10 mM ethanol produced an inhibition of calcium current comparable to that produced by 25 mM ethanol in the absence of the dihydropyridine (Fig. 4). The half-maximum inhibition (IC<sub>50</sub>) of L-type channel open probability by ethanol, calculated by the equation

\[
\frac{I}{I_0} = \frac{1}{1 + \frac{[\text{EtOH}]}{IC_{50}}},
\]

where I is the current amplitude in the presence of ethanol and I<sub>0</sub> is the current in the absence of ethanol, yielded a value of 9.1 mM.

The prolonged openings produced by Bay K made it possible to calculate the distribution of open lifetimes, which was reduced in a concentration-dependent manner by ethanol. In all cases, the data was best fit by the sum of two exponentials (Fig. 5A). The duration of both short and long openings was reduced by
etanol, as can be seen in bar graph form in Figure 5B. Examination of Figure 5A makes clear that ethanol typically exerted a greater effect on the longer openings, since in addition to its effects on open duration, the number of long openings is significantly reduced by higher concentrations of ethanol. Since channel open time was not significantly reduced by 10 mM ethanol, the reduction in open probability seen at this ethanol concentration (Fig. 4) cannot be attributed to an alteration in this parameter, and an additional mechanism must underlie the reduction in open probability. At concentrations of 25 mM and higher, however, the decreases seen in open probability are likely to be due, at least in part, to the reduction in open duration.

The single-channel conductance was unchanged by 25 mM ethanol (Fig. 6), a concentration that had significant effects on channel open time (see Figs. 4, 5). Analysis by all-points histogram of data collected at +10 mV yielded mean single-channel currents of 1.37 ± 0.19 pA in control medium, and 1.35 ± 0.17 pA in the presence of 25 mM ethanol. Channel conductance was unaffected by even higher concentrations of ethanol, as well. In another group of terminals, the mean amplitude of single-channel current was 1.30 ± 0.20 pA in control medium (n = 8) and 1.27 ± 0.20 pA in the presence of 100 mM ethanol (n = 6).

**Nature of the ethanol-channel interaction**

The data obtained in this study can be analyzed in a number of ways to gain insights into the nature of the interaction between ethanol and the calcium channels being inhibited. When the relationship between the reduction of open probability in the Bay K-treated terminals and the ethanol concentration was graphed as a Hill plot, the data yielded a straight line with a
Figure 4. Effects of various concentrations of ethanol on the averaged channel mean open probability ($P_o$) from many different terminals. A. In the absence of Bay K 8644, histograms represent the mean ± SEM $P_o$ in control medium ($n = 9$), 25 mM ethanol ($n = 5$), 50 mM ethanol ($n = 4$), and 100 mM ethanol ($n = 3$). B. In the presence of 5 µM Bay K 8644 added in the pipette solution, histograms represent the mean ± SEM $P_o$ in control medium ($n = 6$), 10 mM ethanol ($n = 2$), 25 mM ethanol ($n = 4$), 50 mM ethanol ($n = 3$), and 100 mM ethanol ($n = 4$). Hill coefficient of 1.045 (Fig. 7), indicating that the interaction between ethanol and the channel does not require cooperativity between multiple binding sites. This is consistent with a single binding site on the channel interacting with a single drug molecule, according to first-order kinetics. If the interaction between a drug and its target is bimolecular, we can calculate its equilibrium dissociation constant, $K_d$, from an analysis of the distribution of open and closed times (Wang et al., 1990a,b; Wang and Lemos, 1992). The modulating effect of ethanol on the Bay K-modified Ca$^{2+}$ channel is considered in terms of a simple kinetic model consistent with the observations:

\[
\text{Closed} \quad \frac{\beta}{\alpha} \quad \text{Open} + \text{Ethanol} \quad \frac{k_{\text{on}}}{k_{\text{off}}} \quad \text{blocked}
\]

The model consists of several open and closed states, where $\alpha$ is the closing and $\beta$ is the opening rate constant for the L-type Ca$^{2+}$ channel. The $k_{\text{on}}$ and $k_{\text{off}}$ represent association and dissociation rate constants of ethanol with the modified channel, respectively. The association of ethanol with the modified Ca$^{2+}$ channel creates a unique nonconducting state, labeled here as "blocked."

In Figure 5, it can be seen that the open time constants of the Bay K-modified channel is decreased by ethanol in a dose-dependent manner. Correspondingly, as shown in Figure 8A, the inverse of the open time constants increased in a linear manner with respect to the ethanol concentration. Therefore, the slope of this plot yields the $k_{\text{on}}$ of ethanol predicted by the above model, according to the equation (McCann and Welsh, 1987)

\[
k_{\text{on}} = \frac{1}{\tau_0 - k_{\text{off}}} [\text{EtOH}]\]

Figure 5. Effects of ethanol on single-channel open time in the presence of Bay K 8644. A, Representative single-channel open time histograms from different terminals in control and various concentrations of ethanol, as indicated, obtained during a voltage pulse to +10 mV ($V_h = -50$ mV). Distribution of open times can be fit with two exponentials, giving the following time constants: Control, $\tau_1 = 1.79$ msec and $\tau_2 = 79.73$ msec; 10 mM ethanol, $\tau_1 = 1.11$ msec and $\tau_2 = 22.32$ msec; 25 mM ethanol, $\tau_1 = 1.02$ msec and $\tau_2 = 16.93$ msec; 50 mM ethanol, $\tau_1 = 0.58$ msec and $\tau_2 = 13.09$ msec; 100 mM ethanol, $\tau_1 = 0.35$ msec and $\tau_2 = 7.86$ msec. B, Single-channel open times averaged from a number of experiments. Bars represent the mean ± SEM channel open times in Control ($n = 6$), 10 mM ethanol ($n = 2$), 25 mM ethanol ($n = 4$), 50 mM ethanol ($n = 4$), and 100 mM ethanol ($n = 5$) of $\tau_1$ (open bar) and $\tau_2$ (hatched bar).
Figure 6. Effects of ethanol on L-type single-channel Ca\(^{2+}\) current amplitude. All-point amplitude histograms of the currents recorded from the same terminal in the presence of 5 μM Bay K (\(V_0 = -50\) mV; \(V_1 = +10\) mV) in the absence (A) and presence (B) of 25 mM ethanol after 5 min. C and O indicate the closed state and open state, respectively. Channel amplitudes, obtained from Gaussian fits, were 1.24 ± 0.21 pA in the absence and 1.28 ± 0.21 pA in the presence of ethanol.

where \(\tau_o\) is the open time constant, \(k_{on}\) is the sum of all rate constants leading to closed but not blocked states, and [EtOH] is the concentration of ethanol. The \(k_{on}\) calculated in this manner is \(1.51 \times 10^4\) M\(^{-1}\) sec\(^{-1}\).

The effect of ethanol was to decrease the mean open time of the Bay K-modified Ca\(^{2+}\) channel, while leaving the distribution of channel closed times unaltered (Fig. 8B). This allows calculation of \(k_{off}\) (141.6 sec\(^{-1}\)) from the equation

\[k_{off} = 1/\tau_c.\]

The equilibrium dissociation constant (\(K_d\)) for ethanol’s interaction with the calcium channel could then be calculated from \(K_d = k_{off}/k_{on}\). The \(K_d\) thus calculated is 9.4 mM, which is very close to the IC\(_{50}\) value (9.1 mM) describing the concentration dependency of the reduction of NP\(_o\) by ethanol in the presence of Bay K 8644.

**Discussion**

The molecular basis for ethanol’s actions has been particularly difficult to elucidate, and very controversial. Much of the literature that examines the biophysical basis for the actions of this drug used extremely high concentrations, above the lethal level in humans, and typically studied model systems not known to be actual targets underlying the effects of ethanol in the body. In this study, we used ethanol levels near those that constitute legal intoxication in humans (22 mM), and we studied vasopressin-releasing nerve terminals involved in some of the behavioral effects of ethanol ingestion. “Whole-cell” patch-clamp studies have shown that 25 mM ethanol significantly inhibits the noninactivating calcium current in the terminals in a manner consistent with this inhibition underlying the reduction of calcium-dependent release of vasopressin from the terminals (Wang et al., 1991b,c). The total macroscopic current that we have recorded previously results from the product of the number of conducting channels, the probability of a channel being open, and the unit current flowing through an individual channel (\(I = NP_o\)). In the present study, we used single-channel recording techniques to establish that the primary action of 25 mM ethanol on the noninactivating calcium channels in the terminal is to decrease their open probability by decreasing their duration of opening. There is no indication that the number of channels operative within the patches included in this study changes as a result of ethanol exposure. Multiple channels within a patch would be obvious during the prolonged openings that occurred in the presence of Bay K, and we did not see evidence of multiple openings, either before or after exposure to ethanol. The unitary current also was not altered in the presence of ethanol. Thus, it is likely that the decrease in \(P_o\), attributable to the reduction of channel open time observed in this study, underlies the reduction of macroscopic current previously reported (Wang et al., 1991b,c). The percentage reduction in open time reported in this article (Fig. 5) does, in fact, correlate well with the reduction of macroscopic noninactivating calcium current, elicited from a holding potential of -50 mV, which we previously reported to occur in the nerve terminals at ethanol concentrations of 25 mM and above, in the absence of dihydropyridines (Fig. 3 in Wang et al., 1991b). The inhibition of slowly inactivating current previously reported to occur in the presence of ethanol concentrations as low as 10 mM, when using a holding potential of -90 mV (Wang et al., 1991b), may be an effect of the decrease in open probability reported in this article, but cannot be explained by a change in channel open time, suggesting that another mechanism explains the inhibition of currents elicited from this holding potential at lower ethanol concentrations.
The basis for the increased sensitivity of channel open probability seen in terminals treated with Bay K 8644 is not clear. The IC$_{50}$ for ethanol inhibition of L-type Ca$^{2+}$ current in terminals can be computed from previously reported macroscopic data collected in the absence of Bay K (Wang et al., 1991c), yielding a value of 36.4 mM, which is in general agreement with the IC$_{50}$ of 23.5 mM, which can be computed from the single-channel records in the absence of Bay K reported in this article (see Fig. 4A). Both of these values are significantly higher than the values obtained in the presence of Bay K. Bay K is thought to bias calcium channels into a state characterized by longer duration openings, the "mode 2" type of activity (Hess et al., 1981), and ethanol's effects may be greater when the channel is in this mode. There is evidence from other drugs, such as local anesthetics, that particular states of the receptor have a higher affinity for drug molecules than do other states (Butterworth and Strichartz, 1990).

Neither the noninactivating component of the macroscopic current (Wang et al., 1991a,b) nor the L-type single channels showed recovery from ethanol's effects within the time frame in which we were able to maintain channel activity (<1 hr). This finding is consistent with observations made in vivo, in which plasma vasopressin levels remained significantly suppressed, even after plasma ethanol levels had returned to control levels (Eisenhofer and Johnson, 1982).

**Mechanism of ethanol action**

Our findings suggest that ethanol interacts with the calcium channel in a bimolecular fashion. The Hill plot of the reduction by ethanol of $P_o$ yielded an "n" very close to 1. Moreover, the calculation of the $K_d$ value obtained by analysis of the channel open and closed time distributions is predicated on a bimolecular interaction, and this presumption was corroborated by the close correlation of the IC$_{50}$ for reduction of the $P_o$ by ethanol.

The nature of the interaction with the channel is not clear at this time. Were ethanol acting as an open channel blocker, we might expect that the prolonged openings in the presence of Bay K 8644 would potentiate ethanol's actions, as did occur. However, since the on rate is orders of magnitude slower than the aqueous diffusion limit, pore plugging from the aqueous medium is unlikely. The slow washout of the inhibition of calcium channels also suggests that ethanol is not acting as a simple open channel blocker. Since the partition coefficient of ethanol approaches 1.0, it is unlikely that the drug is trapped in the lipid environment. The most likely explanation is that ethanol interacts with the open channel, causing an alteration in channel function that outlasts the presence of the drug. More experiments designed specifically to answer this question are necessary before a conclusion can be reached. While it is possible to imagine schemes whereby data such as described in this article could arise from a nonspecific action of ethanol on membrane lipids, a more reasonable conclusion is that there is a specific interaction of the drug with L-type calcium channels.
Conclusions

We conclude from these findings that ethanol inhibits the L-type Ca2+ channels in neurohypophysial nerve terminals by decreasing the channel open probability. This inhibition, at ethanol concentrations of 25 mM or greater, is primarily due to a direct effect on channel open time. Therefore, the basis for the reduction in calcium current, and possibly the associated reduction in vasopressin release observed in the presence of ethanol, derives from direct effects on channel-gating kinetics, rather than effects on the number of channels active or the single-channel conductance.

References


