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Integrated Channel Plasticity Contributes to Alcohol Tolerance in Neurohypophysial Terminals

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ABSTRACT

Short-term ethanol challenge results in the reduction of peptide hormone release from the rat neurohypophysis. However, rats that have been maintained on an ethanol-containing diet for 3 to 4 weeks exhibit tolerance to this effect. Mechanistic underpinnings of this tolerance were probed by examining four ion channel conductances critical for neurohormone release. The voltage-gated L-type calcium channel and the functionally linked calcium-activated BK channel represent a functional dyad. Although these channels show opposite drug responses in the naive terminal (i.e., the L-type Ca^{2+} channel is inhibited whereas the BK channel is potentiated), the effect of long-term alcohol exposure is to decrease sensitivity to the short-term administration of drug in both instances. In addition to the shift in sensitivity, current density increased for the L-type Ca^{2+} current and decreased for the BK current, consistent with a compensatory change. Sensitivity to alcohol was also altered for two other channel types studied. Inhibition of the voltage-gated transient Ca^{2+} current was lessened after long-term treatment. I_A, which is not sensitive to the drug at clinically relevant concentrations in terminals from the naive rat, acquires sensitivity after long-term exposure, representing a potentially novel type of tolerance. However, neither the transient Ca^{2+} current nor I_A shows a change in current density, demonstrating the selectivity of this aspect of tolerance. Overall, these results demonstrate that channel plasticity can explain at least a portion of the behavioral tolerance resulting from changes in sensitivity of peptide hormone release. Furthermore, they suggest that an understanding of tolerance requires the examination of dynamically coupled channel populations.

Tolerance represents a critical element of drug action, as well as an example of neuronal plasticity. Various forms of ethanol tolerance have been described, characterized by their time frame (Kalant, 1998). The molecular underpinnings of tolerance are not yet understood. Typically, studies have used either preparations amenable to exploration at the molecular level, for which the role of the molecules studied are not understood in terms of physiological or behavioral events, or a physiological or behavioral function is examined, for which the underlying molecular components are unclear. The rat hypothalamic-neurohypophysial system provides an ideal model to study the short-term and long-term actions of ethanol. Short-term ethanol challenge blocks the release of arginine vasopressin and oxytocin (OT) from both the intact neurohypophysis and from isolated neurohypophysial terminals (Wang et al., 1991a,b; Knott et al., 2000). The diuretic effect of short-term alcohol exposure exhibits tolerance after prolonged ethanol exposure (Schrier et al., 1979; Crabbe et al., 1981; Pohorecky, 1985).

Excitable cell function requires the dynamic interplay of a variety of ion channels and intracellular signaling pathways. The voltage-gated L-type calcium channel and the BK channel, which is activated by both Ca^{2+} and voltage, represent an interactive dyad, in which Ca^{2+} entry through the voltage-gated calcium channel activates the Ca^{2+}-activated BK channel.

In terminals, the activation of voltage-gated Ca^{2+} channels provides the rise of intracellular Ca^{2+} that triggers hormone release, and activation of Ca^{2+}-activated potassium channels completes a feedback loop in which membrane repolarization terminates release. The biophysical basis of alcohol action on both of these channels has been described in the neurohypophysial terminal, as well as with cloned channels in expression systems and planar bilayers (Wang et al., 1994; Dopico et al., 1996, 1998, 1999a; Chu et al., 1998). Ethanol inhibits the L-type channel and potentiates the BK channel (Wang et al., 1991a,b, 1994; Dopico et al., 1996). These actions produce the reduction of peptide hormone release that follows ethanol ingestion. In both channels, ethanol modulates the gating properties of the channel, leaving parame-

ABBREVIATIONS: OT, oxytocin; BK, calcium-activated potassium channel; VGCC, voltage-gated calcium channels; I_{Ca}, transient calcium current; I_A, potassium A-current; ELISA, enzyme-linked immunosorbent assay; HEDTA, *N*-hydroxy-EDTA; TEA, tetraethylammonium; 4-AP, 4-aminopyridine; ANOVA, analysis of variance; HP, holding potential.

ters such as ion-selectivity and voltage-sensitivity unaffected (Wang et al., 1994; Dopico et al., 1996, 1998). Voltage-gated calcium channels (VGCC) and calcium-activated K^+ channels are colocalized in a number of preparations (Marrion and Tavalin, 1998), such that Ca^{2+} influx through the VGCC preferentially activates the associated Ca^{2+}-activated potassium channel. It is tempting to postulate that alterations in one of these channel populations after long-term drug exposure is accompanied by alterations in the other. Thus, we decided to explore the response of this dyad of channels to short-term ethanol in terminals from rats with long-term exposure to ethanol.

We also monitor two other channels critical for control of hormone release from neurohypophysial terminals, one of which (transient I_{Ca}) is only moderately sensitive to the short-term action of the drug in the naive terminal (Wang et al., 1991b) and another (I_A), which is insensitive to ethanol in the naive terminal (Dopico et al., 1996).

Materials and Methods

Animals

Male Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing 250 to 300 g were raised on a liquid diet (Research Diets, Inc., New Brunswick, NJ) as described previously (Knott et al., 2000). Briefly, the long-term treatment group and the control animals were isocalorically yoked, and the alcohol content of the diet of the long-term ethanol animals was incrementally increased over the course of 4 days. Although initial exposure to the EtOH-containing diet leads to reduced food consumption, within two weeks, intake returns to levels observed before introduction of EtOH to the diet. In addition, blood alcohol levels peaked between days 9 and 13 of the diet, then declined to stable values of 30 to 35 mM by day 24 (Knott et al., 2000).

Preparation. The neurohypophysis was removed from the animal within 1 min of sacrifice and placed in low-calcium (~3 μM) Locke's solution (see *Solutions*). The pars intermedia was dissected away and discarded. Neurohypophysial terminals were isolated as described previously (Lemos and Nordmann, 1986). The dissociated terminals were first placed within a plastic ring centered in a sterile polystyrene dish. The surrounding dish was then filled with low-calcium Locke's solution. The ring was removed from the dish after 1 min and the terminals allowed to sit for 3 min before a slow perfusion (1 ml/min) with 2.2 mM calcium Locke's for 3 to 5 min, followed by a fast perfusion (8 to 10 ml/min) for a minimum of 10 min. This allows the terminals to adhere to the bottom of the dish but remain attached loosely enough to be lifted from the bottom after formation of a 1-GΩ seal. The dissociated terminals were 6 to 12 μm in diameter and easily identified using phase and interference (Hoffmann) optics. The terminals were unexposed to alcohol during these procedures for 30 to 150 min, until short-term challenge. Thus, the terminals may be considered to be in the initial stages of withdrawal when short-term EtOH challenge is begun.

Hormone Release

Hormone collection from isolated neurohypophysial terminals was done as described previously in Knott et al. (2000). Briefly, rat neurohypophyses were homogenized, and the homogenate was centrifuged at 2400*g* for 6 min. The resulting pellet contains highly purified nerve terminals. These nerve terminals were loaded equally onto four filters (0.45-μm Acro disc; Gelman Sci., Ann Arbor, MI) and perfused at 37°C with normal Locke's medium. Terminals were rinsed with Locke's medium containing 0.02% (w/v) bovine serum albumin for 45 min, followed by 0 Na^+ Locke's (normal Locke's solution with an equimolar concentration of *N*-methyl-D-glucamine-chloride replacing the 145 mM Na^+, (with 0.02% bovine serum albumin) for 15 min. All buffer solutions were 305–310 mOsm. Depolarization-coupled release was stimulated with high K^+ (50 mM) as described in Cazalis et al. (1987). The concentration of *N*-methyl-D-glucamine-chloride was reduced when high K^+ was used in the perfusion. Fractions were collected at 2-min intervals during the following sequence of solution changes: 0 Na^+ Locke's (10 min); 0 Na^+ Locke's containing 50 mM K^+ (4 min); 0 Na^+ Locke's (20 min); 0 Na^+ Locke's containing 75 mM EtOH (4 min); and 0 Na^+ Locke's, 50 mM K^+, and 75 mM EtOH (matching the previous exposure) (4 min). Finally, fraction collection was continued during perfusion with 0 Na^+ Locke's (20 min), followed by 0 Na^+ Locke's containing 50 mM K^+ (4 min), to determine possible hormone store depletion or residual EtOH effects. The samples were frozen and stored at −80°C for quantitative analysis by ELISA.

Hormone Assay

Released oxytocin (OT) was measured by assaying 30 μl from every 250-μl fraction collected during each experiment with an ELISA kit (Assay Designs, Inc., Ann Arbor, MI). The sensitivity limit of the assay was 1 pg and cross-reactivity of OT for vasopressin (the other neurohypophysial peptide) was <0.001%.

Electrophysiology

Whole-Cell Recordings. Potassium currents were obtained from dissociated terminals using the whole-cell patch-clamp technique. The terminal was lifted off the bottom of the dish and placed into a "sewer pipe" perfusion stream containing the appropriate solution. Currents were recorded using a patch-clamp amplifier (Axopatch 200B; Axon Instruments Inc., Union City, CA) at a bandwidth of 5 kHz and leak-subtracted off-line. Data were acquired and analyzed with PClamp6 software (Axon Instruments). BK current amplitude was measured during the current plateau, 100–400 ms after the beginning of the voltage step. I_A amplitude immediately after the peak was obtained from the average current between 5 and 20 ms after the beginning of the voltage step. Electrodes (David Kopf Instruments, Tujunga, CA) were pulled from 100-μl glass pipettes (Drummond Scientific Co., Broomall, PA). The electrode shanks were coated with Sylgard (Dow Corning Co., Midland, MI) to reduce capacitance, and the tips fire-polished on a microforge (Narashige, Tokyo, Japan) to give a resistance of 4 to 8 MΩ when filled with pipette solution (see *Solutions*).

Perforated Patch Recordings. Ca^{2+} current recordings were obtained using the perforated-patch technique (Wang et al., 1992). After isolating the terminals as mentioned above and rinsing with normal Locke's solution, the terminals are further rinsed with 5 mM barium (in place of calcium) Locke's for 5 min and all subsequent current acquisition performed in the barium Locke's. Perforation of the terminals was obtained by the addition of amphotericin B to the pipette solution (see *Solutions*).

Onset and reversibility of alcohol effects. For each of the channels examined, the short-term effects of ethanol were evident within 5 s of exposure and the effects were reversed within 30 s of washout of the drug, for both naive terminals and those with long-term exposure.

Solutions. For conventional whole-cell recordings, the pipette solution consisted of 120 mM potassium gluconate, 10 mM HEPES, 20 mM *N*-methyl-D-glucamine chloride, 15 mM KCl, 2.25 mM $CaCl_2$, 2.65 mM $MgCl_2$, 2 mM EGTA, 2 mM HEDTA, 0.2 mM cAMP, 2 mM Mg-ATP, pH 7.3 and 315 mOsM (~ 4 μM free Ca^{2+} concentration) (Fabiato, 1988). Calcium and HEDTA are omitted in the 0-calcium experiments.

In all whole-cell recordings, the terminals were bathed in Locke's solution consisting of 130 mM NaCl, 15 mM glucose, 10 mM HEPES, 5 mM KCl, 2.2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.3 and 305 mOsM. In low-calcium (3 μM) Locke's, the 2.2 mM $CaCl_2$ was reduced to 1.96 mM and 2 mM EGTA is added. For the 0-calcium experiments, 5 mM

$BaCl_2$ was used in place of 2.2 mM $CaCl_2$. An osmotic difference of 10 mOsM was maintained between the pipette and bath solutions to enhance seal formation.

For perforated patch recordings, the pipette solution consisted of 130 mM CsGlut, 15 mM $CsCl_2$, 5 mM glucose, 5 mM tetraethylammonium (TEA) chloride, 1 mM $MgCl_2$, ~300 μM amphotericin B, pH 7.3 and 315 mOsM. In all perforated patch recordings, the terminals were bathed in Locke's solution followed by a 5 mM barium Locke's solution, which consisted of 130 mM NaCl, 10 mM glucose, 10 mM HEPES, 5 mM KCl, 5 mM $BaCl_2$, 2 mM $MgCl_2$, pH 7.3 and 305 mOsM. Long-lasting L-type calcium currents were enhanced by 1 μM Bay K and blocked by 2.5 μM nicardipine in "sewer pipe" perfusions.

Chemicals

Ethanol, HEPES, and $MgCl_2$ were obtained from American Bioanalytical (Natick, MA). $BaCl_2$ and $CaCl_2$ were from Fisher Scientific (Fair Lawn, NJ). Potassium gluconate, glucose, *N*-methyl-D-glucamine, HEDTA, EGTA, TEA-chloride, 4-aminopyridine (4-AP), charybdotoxin, Bay K, cAMP, and Mg-ATP were obtained from Sigma-Aldrich Chemical (St. Louis, MO). NaCl and KCl were from EM Science (Gibbstown, NJ).

Statistical Analysis

All values in this study are reported as mean ± S.E.M. We evaluated the differences between the naive groups and those with long-term ethanol exposure groups using ANOVA, as described in the legends to Figs. 3 to 7. Statistical significance for all analyses was set at $p < 0.05$.

Results

Tolerance to Ethanol Inhibition of Peptide Hormone Release. Fig. 1 shows representative data contrasting the short-term ethanol inhibition of oxytocin release in isolated neurohypophysial terminals from an alcohol-naive rat with the absence of inhibition in terminals from a rat with long-term exposure. Similar tolerance is observed with vasopressin release, both in the intact neurohypophysis and in isolated terminals (Knott et al., 2000). To determine the underlying mechanism for this tolerance of peptide release, pharmacological blockers and voltage protocols were used to isolate and study the effects of drug history on the four main membrane conductances that control the release of neurohormones from neurohypophysial terminals and for which the degree of short-term sensitivity to ethanol has been determined previously. These currents were 1) the voltage-gated L-type Ca^{2+} current (Wang et al., 1991a,b), 2) the calcium-activated potassium (BK) current (Dopico et al., 1996), 3) the voltage-activated transient Ca^{2+} current, which is a mixture of N, R, and Q subtypes of Ca^{2+} channel (Wang et al., 1992, 1997, 1999), and 4) the fast, transient, potassium A-current (I_A) (Dopico et al., 1996). BK and I_A represent the predominant outward currents in neurohypophysial terminals (Thorn et al., 1991; Wang et al., 1992) (see Fig. 2).

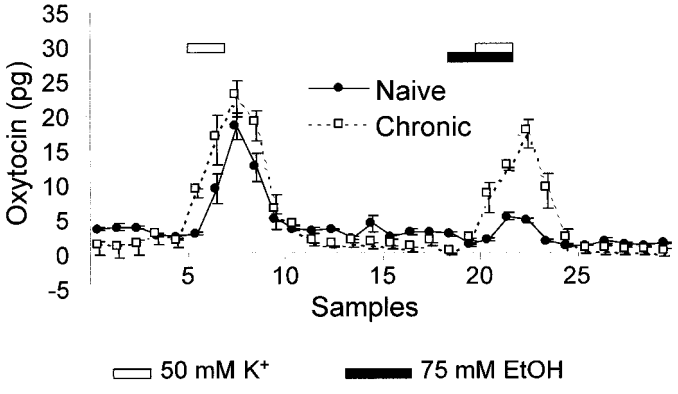

Fig. 1. Representative examples of OT release, determined by ELISA, from an isolated neurohypophysial terminal preparation challenged with ethanol. Data are representative of mean ± S.E.M. of four filters (see *Materials and Methods*). Ethanol reduces high K^+ evoked hormone release in terminals taken from naive animals (solid line). However, hormone release from terminals isolated from rats with long-term exposure is far less sensitive to ethanol inhibition (dotted line). Although the magnitude of hormone release during the initial high K^+ stimulation differs in these two examples, this parameter did not consistently vary with long-term exposure, and differences in the effects of ethanol challenge were independent of the magnitude of release during the initial depolarization. Absolute values of release cannot be reliably interpreted because of variation in the number of terminals trapped on the filter in each experiment.

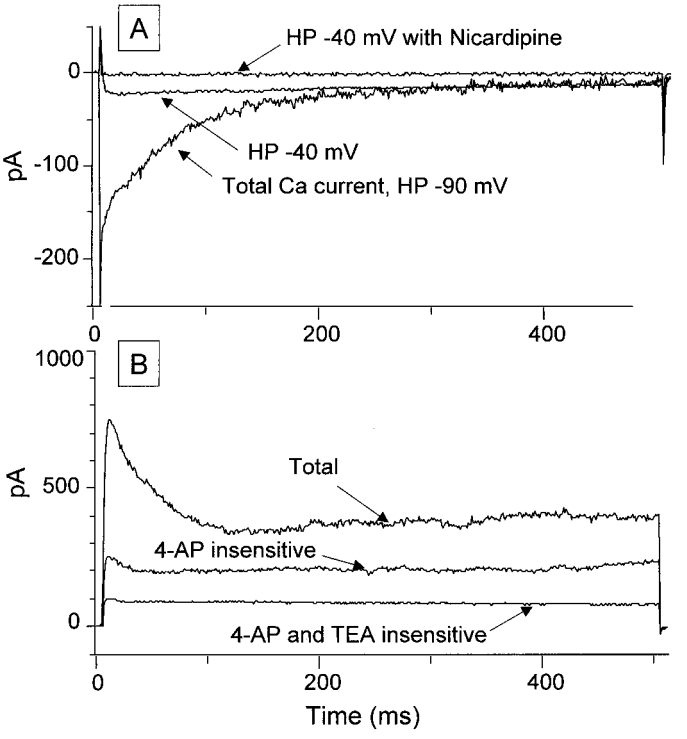

Fig. 2. Current separation in a representative isolated neurohypophysial terminal. A, currents through Ca^{2+} channels. The bottom trace (holding potential, −90 mV; voltage step to 0 mV for 500 ms) recorded in normal Locke's solution containing 5 mM barium, represents the total calcium current. Isolation of the transient current was obtained by inhibiting the long-lasting portion with nicardipine, an L-type calcium channel blocker. The middle current trace represents the L-type current, obtained by clamping the terminal membrane at −40 mV (where the transient current is inactivated) and stimulating with a voltage step to + 10 mV for 500 ms. The upper trace confirms the identity of the −40mV holding potential (HP) trace as uncontaminated L-type current, by demonstrating its blockade after the addition of 2.5 μM nicardipine. In all traces, barium is the carrier ion. B, potassium currents. The terminal membrane was clamped at −80 mV and stimulated with a voltage step to + 40 mV for 500 ms. The upper trace is the total current, recorded in normal Locke's solution containing 2.2 mM calcium. Addition of the I_A channel inhibitor 4-AP (7 mM) removes the fast inactivating I_A, leaving a combined non-inactivating BK current and a resistant current (middle trace). BK is blocked by 100 mM TEA-chloride, a potassium channel blocker, leaving the resistant current (lower trace) which is insensitive to the potassium channel blockers. In subsequent figures, I_A is isolated by subtracting the 4-AP insensitive current from the total current and BK current is isolated by subtracting the TEA-chloride–resistant current from the 4-AP–insensitive current. Traces are the average currents from four identical voltage steps in the same terminal.

L-Type Ca^{2+} Current. This neurohypophysial terminal current has previously been shown to be significantly blocked by intoxicating concentrations of ethanol (Wang et al., 1991a,b), and this is confirmed in the present study. Representative traces of the response to short-term 25 mM ethanol challenge are shown in Fig. 3A, in which the reduced action of the drug in terminals removed from rats with long-term exposure is evident. A full concentration-response relationship is shown in the bar graph in Fig. 3B, demonstrating the decreased sensitivity of the L-type current, indicative of the development of tolerance.

BK Current. Short-term ethanol exposure confirmed previous findings that BK current in the terminal is augmented by intoxicating levels of ethanol (Dopico et al., 1996) and provides evidence that these channels are less sensitive to short-term potentiation in terminals from rats with long-term exposure to the drug (Fig. 4A). To verify that the potentiated current was BK, we replaced the calcium in the perfusion medium with barium. Ethanol did not potentiate the remaining current (data not shown), confirming the identity of the potentiated current as BK current. The concentration-response relationship generated by short-term exposure to varying concentrations of ethanol indicates that potentiation of BK currents is shifted along the concentration axis in a manner indicative of decreased sensitivity after long-term exposure (Fig. 4B).

Transient Ca^{2+} current. This current has previously been shown to be reduced by short-term ethanol exposure, although less so than the L-type Ca^{2+} channel (Wang et al., 1991a,b). As with the L-type current, a representative trace shows a significant reduction in the short-term sensitivity of the channel in terminals removed from rats with long-term exposure (Fig. 5A), and the concentration-response relationship indicates a decreased sensitivity of the channel over a range of short-term challenge concentrations (Fig. 5B).

I_A. The fast, transient current, I_A, is the other prominent outward current in the terminal; this current has previously been shown to be insensitive to short-term ethanol challenge at intoxicating levels of the drug (Dopico et al., 1996). Thus, we were able to compare plasticity in this potassium channel population with that of the more sensitive BK channel. Interestingly, suppression of I_A by short-term ethanol challenge at intoxicating levels is observed *only* after long-term exposure (Fig. 6).

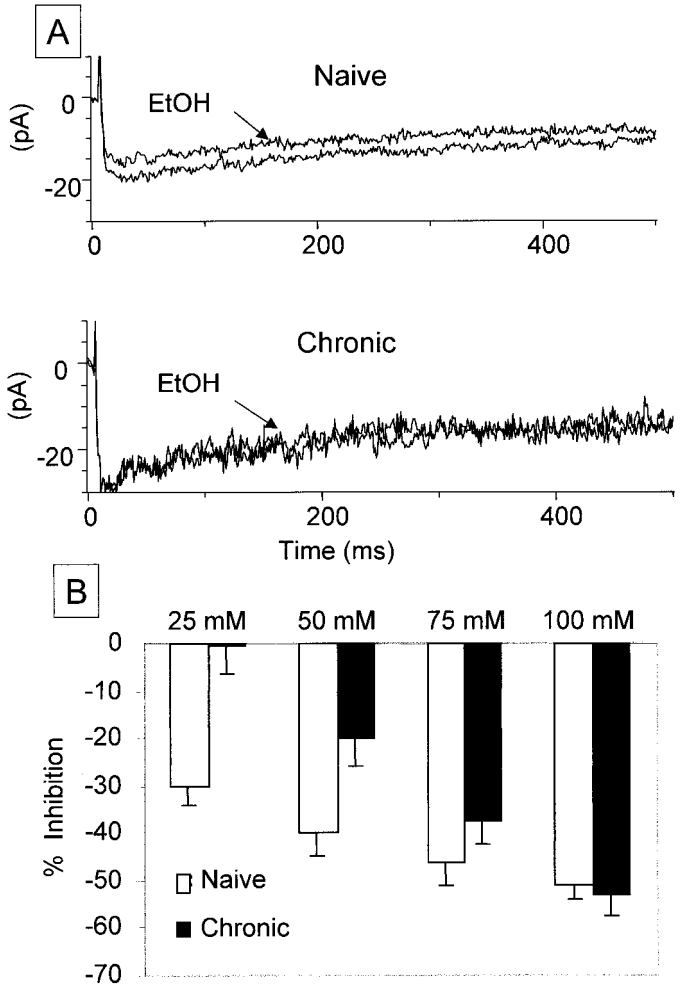

Fig. 3. A, representative L-type calcium current traces from naive terminals (above) and those with long-term exposure (below) challenged with 25 mM ethanol. HP, −40 mV; step to +10 mV. Bay K (1 μM) was present to enhance L-type current. Any current remaining after introduction of 2.5 μM nicardipine, an L-type calcium channel blocker, is subtracted. B, dose-dependent response to a series of short-term ethanol challenges as in A. Measurements were the averaged current between 100 and 400 ms (n = 3–4). Two-way ANOVA of L-type calcium channel inhibition across levels of EtOH reveals that naive animals respond differently than animals with long-term exposure depending upon levels of EtOH; $F_{3,19} = 3.49$ $p = 0.036$. Once EtOH levels have reached 100 mM, differences between naive animals and animals with long-term exposure becomes nonsignificant ($q_{1,23} = 0.40$; $p > 0.05$), possibly because the high degree of inhibition at this concentration leads to small currents subject to greater noise in the data.

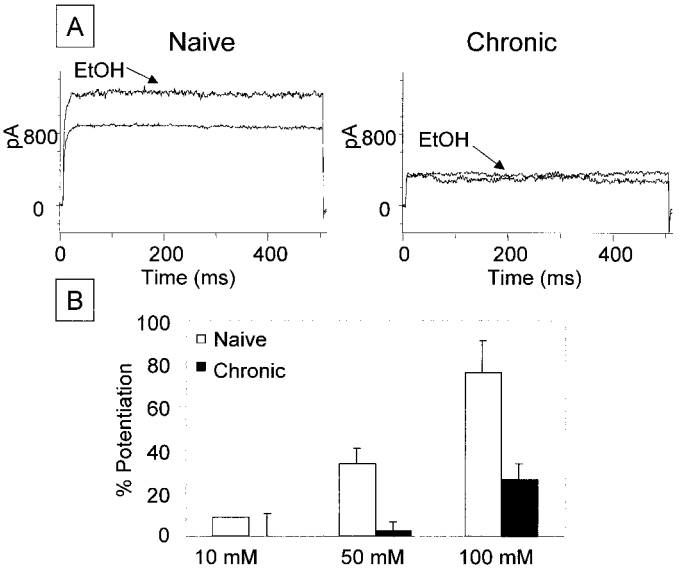

Fig. 4. A, representative BK current traces from terminals challenged with 50 mM ethanol. Holding potential, −80 mV; step to +40 mV in the presence of 7 mM 4-AP, an I_A channel blocker (see Fig. 2). B, concentration-dependent response to a series of ethanol challenges. Measurements were the averaged current between 100 and 400 ms after the start of the depolarizing pulse; current remaining after treatment with 100 mM TEA-chloride was subtracted (n = 3–4). Two-way ANOVA of BK potassium channel potentiation reveals a significant difference between naive versus long-term groups ($F_{1,13} = 19.24$; $p = 0.001$) and EtOH concentration levels ($F_{2,13} = 15.27$; $p < 0.001$), but no group by EtOH concentration interaction ($F_{2,13} = 2.89$; $p > 0.05$). Pairwise analysis using Tukey's honestly significant difference test α adjustment reveals that naive animals have a significantly higher BK current potentiation than do animals with long-term exposure, regardless of EtOH level ($q_{1,17} = 5.60$; $p = 0.03$).

Current Kinetics. We focused on the BK current to test whether basic parameters of channel function were altered concurrent with the observed shift in ethanol sensitivity. Such changes might provide insight into the basis for the shift in sensitivity, because different isoforms of the single *slo* gene generating the channel-containing BK α subunit, or association with one of the auxiliary β subunits, is known to alter such parameters as activation kinetics and calcium and toxin sensitivity of the BK channel (Wallner et al., 1999; Brenner et al., 2000). Neither the activation kinetics nor the voltage-dependence of the BK current (data not shown) was altered as a result of long-term exposure. Kinetics and voltage-dependence of I_A were also unaffected by long-term ethanol.

Current Density. In addition to the tolerance conferred by the changes in response to short-term ethanol challenge in the four channels monitored, adaptive changes might also include an alteration in the number of channels or conduction properties of the channels to counteract the potentiation or inhibition of current observed with short-term ethanol in the naive animal. Such changes have been noted for L-type voltage-gated Ca^{2+} channels, which are up-regulated in response to long-term drug exposure in a number of preparations (Messing et al., 1986; Littleton et al., 1992; Grant et al., 1993; Gerstin et al., 1998). We used capacitance measurements to determine the membrane area of the terminals, and then calculated the current density in naive versus terminals with long-term exposure. Capacitance was correlated with the diameter of the terminal, and this relationship was unaltered by drug treatment, suggesting that infolding was not induced by drug treatment (Fig. 7A). However, L-type Ca^{2+} and BK current density were both significantly altered, but in a reciprocal manner in terminals obtained from the rats on the alcohol diet: L-type current was up-regulated and BK currents down-regulated, consistent with this mechanism of tolerance. In contrast, there was no statistically significant change in channel density apparent for either the transient Ca^{2+} current or I_A after long-term exposure (Fig. 7B). Thus,

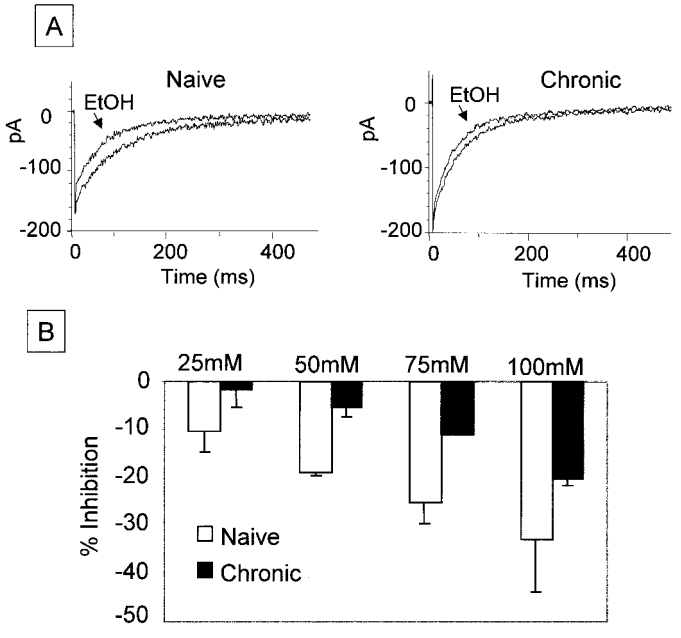

Fig. 5. A, representative transient calcium current traces from terminals challenged with 75 mM ethanol. Holding potential, −90 mV; step to +0 mV in the presence of 2.5 μM nicardipine. B, dose-dependent response to a series of ethanol challenges. Measurements were taken at the peak of transient I_{Ba} (n = 3–4). Two-way ANOVA of transient calcium channel inhibition reveals a significant difference between naive versus long-term groups ($F_{1,17}$ = 25.23; $p < 0.001$) and EtOH concentration levels ($F_{3,17}$ = 19.04; $p < 0.001$), but no group by EtOH interaction ($F_{3,17}$ = 0.67; $p > 0.05$). Pairwise analysis using Tukey's honestly significant difference test α adjustment reveals that naive animals have a significantly higher transient calcium current inhibition than do animals with long-term exposure, regardless of EtOH concentration. ($q_{1,23}$ = 8.10; p = 0.009). Traces are the average currents from four identical voltage steps in the same terminal.

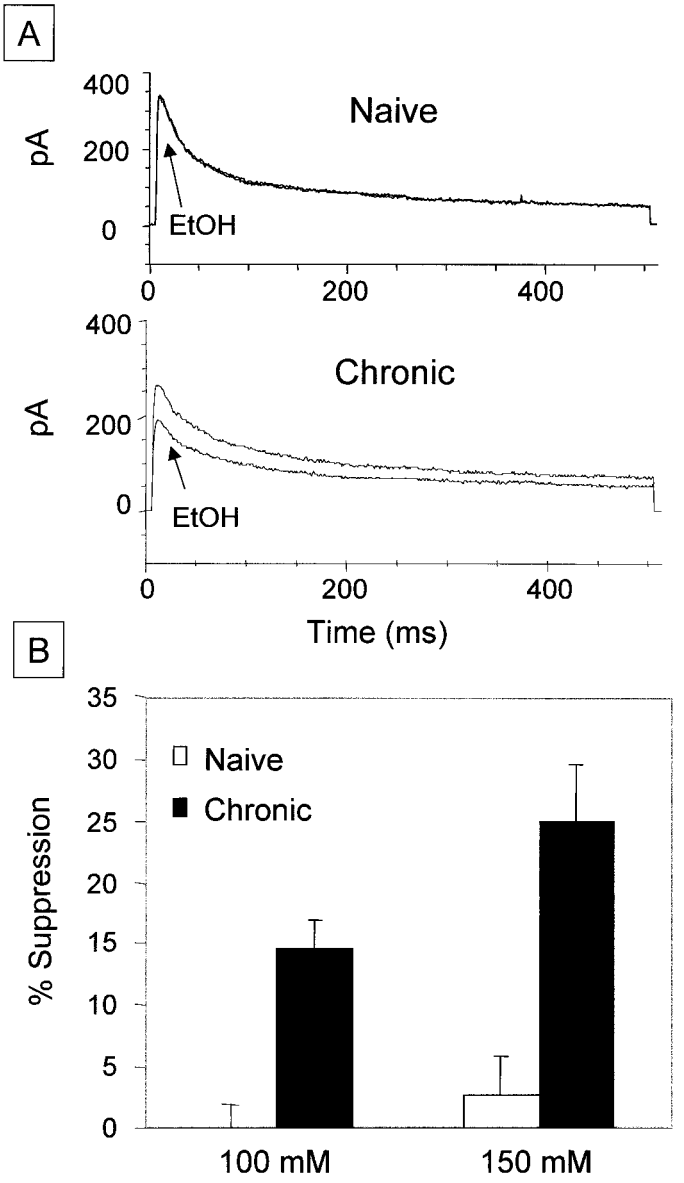

Fig. 6. A, representative I_A traces from terminals challenged with 150 mM ethanol. HP, −80 mV; step to +40 mV. B, concentration-dependent response to ethanol challenge. Measurements were the average current between 5 and 20 ms after the start of the depolarizing pulse (TEA-chloride–resistant current subtracted; n = 3–4). Two-way ANOVA of I_A suppression reveals a significant difference between naive versus long-term groups ($F_{1,8}$ = 36.10; $p < 0.001$), but no difference in effect for EtOH concentration ($F_{1,8}$ = 4.49; $p > 0.05$) nor group by EtOH interaction ($F_{1,8}$ = 1.81; $p > 0.05$). Naive animals have a significantly lower I_A potassium channel suppression than do animals with long-term exposure, regardless of EtOH level. Traces are the average currents from four identical voltage steps in the same terminal.

drug-induced change in current density reflects a channel-specific effect on this parameter.

Recovery. The persistence of the changes in drug sensitivity produced by long-term ethanol was examined for the BK current. Fourteen days after rats have been returned to an ethanol-free diet, the short-term potentiation of BK current in terminals has returned to values observed in ethanol-naive rats (Fig. 8).

Discussion

The findings reported here support the idea that changes in the molecular underpinnings of an ethanol-tolerant behavior can be identified. The four ionic currents examined in this study play a major role in shaping the action potential and in spike patterning, both of which control the Ca^{2+} entry into the terminal important for the release of neuropeptides (Hotson and Prince, 1980; Wong and Prince, 1981; MacDermott and Weight, 1982; Bondy et al., 1987). Our understanding of the biophysical basis for ethanol's short-term action on membrane channels is well formulated for the L-type Ca^{2+} channel and the BK channel. For both, ethanol modulates the gating properties of the channel, leaving other parameters such as ion selectivity and voltage-dependence unaffected (Wang et al., 1994; Dopico et al., 1996). Interestingly, isoforms of the BK gene product are differentially inhibited by ethanol (Dopico et al., 1998; Walters et al., 2000). Moreover, whereas the neurohypophysial terminal BK channel is potentiated by intoxicating levels of ethanol, the BK channel in the associated hypothalamic cell body is unaffected by the drug at these concentrations (Dopico et al., 1999b). Potentiation of BK current (Madsen and Edeson, 1990; Jakab et al., 1997) and inhibition of L-type Ca^{2+} current (Wang et al., 1991b; Mullikin-Kilpatrick and Treistman, 1995; Dopico et al., 1998; Widmer et al., 1998; Calton et al., 1999) by short-term ethanol challenge have been seen in a number of preparations. Potentiation of BK current has also been observed for cloned channels expressed in oocytes (Dopico et al., 1998), and in channels removed from native membrane and reconstituted into planar lipid bilayers (Chu et al., 1998), all suggesting that the drug interacts directly with the channel protein.

A number of mechanisms may underlie the changes in ethanol sensitivity that we observed for each of the channels. For purposes of discussion, we focus on the BK channel, although similar possibilities apply to the other channel types. Because isoforms of the *slo* gene encoding the channel-forming α subunit or association of the α subunit with one of the four currently-identified β subunits produces BK channels with varied characteristics (Adelman et al., 1992; Wallner et al., 1999; Xia et al., 1999; Brenner et al., 2000), genetic alterations in subunit composition might underlie the decreased sensitivity observed. By 14 days after rats have been returned to an ethanol-free diet, the short-term potentiation of BK current in terminals has returned to values observed in

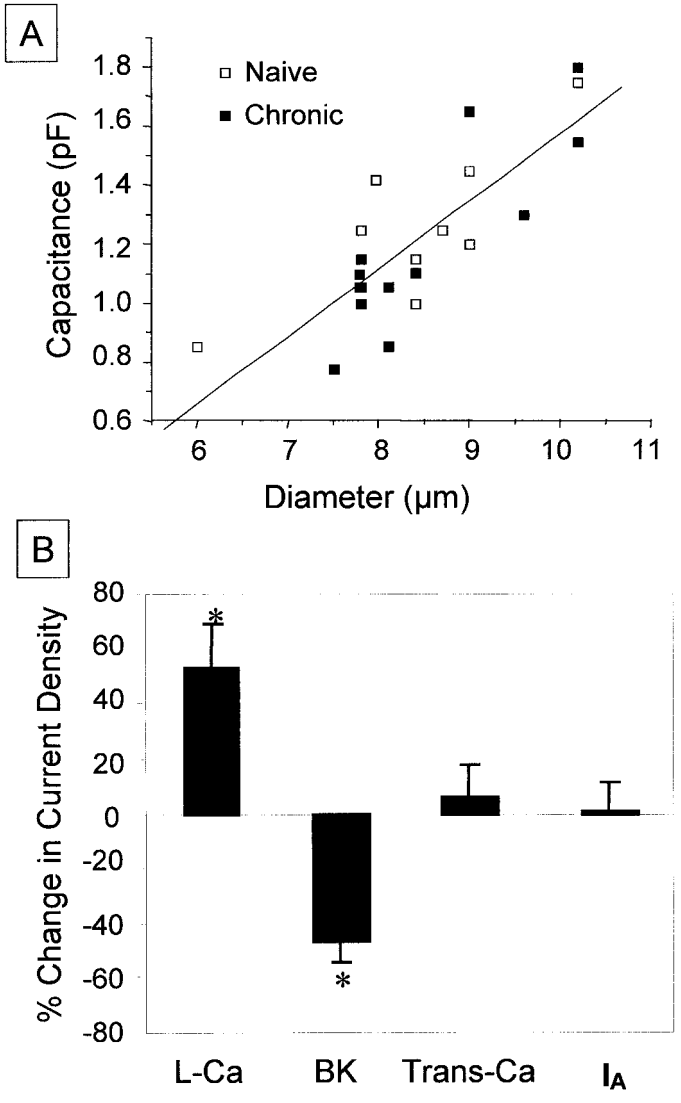

Fig. 7. A, Terminals from naive animals (□) and those with long-term exposure (■) showed a similar linear relationship between size and capacitance. B, L-type calcium and BK current densities (current/capacitance) were significantly different in the terminals from animals with long-term exposure ($n = 14$) compared with those from naive animals ($n = 12$) *, $p < 0.05$. There was no comparable change in either the transient Ca^{2+} current or I_A as a function of long-term ethanol exposure.

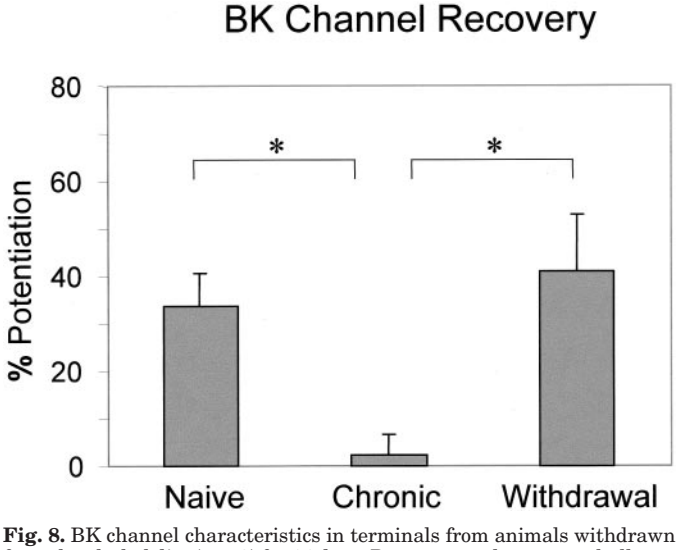

Fig. 8. BK channel characteristics in terminals from animals withdrawn from the alcohol diet ($n = 3$) for 14 days. Response to short-term challenge with 50 mM EtOH. The membrane was clamped at −80 mV and stepped to +40 mV. Data for naive and animals with long-term exposure are from Fig. 4. One-way ANOVA of the potentiation of BK channels reveals a significant group difference ($F_{2,7} = 4.58$; $p = 0.05$) with naive and withdrawal groups potentiated at a higher level than the animals with long-term exposure ($n = 4$).

ethanol-naive rats. Because association of many of the β subunits alter activation kinetics and calcium-dependence, the lack of change in voltage-sensitivity and activation kinetics make it unlikely that some of these associations are responsible for tolerance. Alternatively, post-translational modifications, such as phosphorylation-dephosphorylation can significantly affect the ethanol-sensitivity of BK current (Jakab et al., 1997). Finally, changes in lipid composition of the membrane could affect channel characteristics. BK channels reconstituted into planar lipid bilayers are modulated by cholesterol (Chang et al., 1995), and recent evidence suggests that potentiation of reconstituted channels by short-term ethanol is also modulated by cholesterol content (J. Crowley, S. Treistman, and A. Dopico, in preparation). A large body of literature describes altered membrane lipid composition, including cholesterol levels, in a variety of tissues after long-term ethanol exposure (Chin and Goldstein, 1981; Wood et al., 1989; Ho et al., 1994).

Functionally, the change in BK and L-type current density observed would counteract the potentiation and inhibition produced by short-term drug action, compatible with the development of tolerance. It is not clear whether this reflects regulation of the number of pre-existing channels, or a change in individual channel properties, such as a reduction or augmentation of single channel conductance. This question will require the use of single-channel recording techniques to answer. A combination of labeling and electrophysiological techniques has demonstrated that L-type calcium channels in PC-12 cells, which are inhibited by short-term ethanol challenge, are up-regulated by the addition of new channels after long-term ethanol exposure (Messing et al., 1986; Grant et al., 1993). The fact that I_A and transient Ca^{2+} current show changes in sensitivity independent of changes in current density suggests that these processes are controlled by distinct mechanisms.

The response of I_A to long-term drug treatment differed from that of the other currents measured in that although I_A was insensitive to ethanol at intoxicating levels in the naive rat, it exhibits enhanced sensitivity after long-term exposure, which is counterintuitive to a concept of tolerance in which a reduced response is anticipated. Our results make it clear that plasticity in response to long-term drug exposure differs among channel types. Interestingly, although the short-term sensitivity gained by the I_A channel still occurs at concentrations above those typically seen in the naive user, it becomes more meaningful in the context of a long-term drug user, in which higher ethanol concentrations are tolerated.

Each of the currents examined is an important determinant of action potential shape and patterning (Hotson and Prince, 1980; Wong and Prince, 1981; MacDermott and Weight, 1982). Our results confirm that tolerance at the channel level represents an integrated response among interacting populations. This is most apparent for the most sensitive channels, the L-type calcium channel and the BK channel: although they show opposite drug responses in the naive terminal (i.e., the VGCC is inhibited whereas the BK channel is potentiated), the effect of long-term drug exposure is to decrease sensitivity to the short-term administration of drug in both instances, and the current density of the calcium channel is up-regulated, whereas that of BK is down-regulated. This integrated response to drug exposure is particularly important in maintaining the balanced influence of this channel dyad on release. An interesting possibility is that integrated changes in channel populations are coordinated by control elements at the genetic or other level, such that the consequences of long-term drug exposure result from actions at this level rather than from independent actions upon each of the distinct channel populations. A complete picture of the role of channel plasticity in ethanol tolerance to short-term inhibition of hormone release will require inclusion of changes in channel properties in the hypothalamic cell bodies of these neurons, which play a critical role in the generation of patterning, as well as alterations in intracellular calcium dynamics in the terminals. This model system, in which release and channel activity may be viewed concomitantly, provides a unique opportunity to understand the molecular basis for drug tolerance.

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