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Acute Alcohol Tolerance Is Intrinsic to the BK_{Ca} Protein, but Is Modulated by the Lipid Environment*

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Ethanol tolerance, in which exposure leads to reduced sensitivity, is an important component of alcohol abuse and addiction. The molecular mechanisms underlying this process remain poorly understood. The BK_{Ca} channel plays a central role in the behavioral response to ethanol in *Caenorhabditis elegans* (Davies, A. G., Pierce-Shimomura, J. T., Kim, H., VanHoven, M. K., Thiele, T. R., Bonci, A., Bargmann, C. I., and McIntire, S. L. (2003) *Cell* 115, 655–666) and *Drosophila* (Cowmeadow, R. B., Krishnan, H. R., and Atkinson, N. S. (2005) *Alcohol. Clin. Exp. Res.* 29, 1777–1786). In neurons, ethanol tolerance in BK_{Ca} channels has two components: a reduced number of membrane channels and decreased potentiation of the remaining channels (Pietrzykowski, A. Z., Martin, G. E., Puig, S. I., Knott, T. K., Lemos, J. R., and Treistman, S. N. (2004) *J. Neurosci.* 24, 8322–8332). Here, heterologous expression coupled with planar bilayer techniques examines two additional aspects of tolerance in human BK_{Ca} channels. 1) Is acute tolerance observed in a single channel protein complex within a lipid environment reduced to only two lipids? 2) Does lipid bilayer composition affect the appearance of acute tolerance? We found that tolerance was observable in BK_{Ca} channels in membrane patches pulled from HEK cells and when they are placed into reconstituted 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylserine membranes. Furthermore, altering bilayer thickness by incorporating the channel into lipid mixtures of 1,2-dioleoyl-3-phosphatidylethanolamine with phosphatidylcholines of increasing chain length, or with sphingomyelin, strongly affected the sensitivity of the channel, as well as the time course of the acute response. Ethanol sensitivity changed from a strong potentiation in thin bilayers to inhibition in thick sphingomyelin/1,2-dioleoyl-3-phosphatidylethanolamine bilayers. Thus, tolerance can be an intrinsic property of the channel protein-lipid complex, and bilayer thickness plays an important role in shaping the pattern of response to ethanol. As a consequence of these findings the protein-lipid complex should be treated as a unit when studying ethanol action.

The lipid hypothesis of alcohol action (4), in which the actions of ethanol on neuronal membranes was thought to be explicable mainly in terms of actions on membrane lipids, secondarily affecting proteins, has largely been replaced by the protein hypothesis.

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This latter is predicated on findings that alcohol can interact directly with membrane proteins to affect function (5–11). Of course, the focus on alcohol-protein interaction does not preclude a role for membrane lipids in modulating this interaction. Indeed, recent studies have made it clear that alterations in lipid environments can alter the response of signaling proteins such as membrane ion channels (12) to alcohol (13, 14). Behaviorally, tolerance to the continued presence of ethanol and other drugs of abuse plays an important role in the development of dependence and addiction. In fact, the strength of acute behavioral tolerance in naïve humans is one of the better known predictors of the development of alcohol addiction (15). The elucidation of the molecular mechanisms underlying tolerance and other forms of drug-related neuroadaptation is an important goal of current research in the addiction field.

Large conductance Ca²⁺ and voltage-gated K⁺ (BK_{Ca}) channels (16) play an important role in the regulation of neuronal excitability, cell volume regulation (17), excitation-contraction coupling, and hormonal secretion (18–20). Recently, it has been demonstrated that the BK_{Ca} channel may be a direct target for ethanol (21) and may play a central role in the behavioral response to ethanol in *Caenorhabditis elegans* (1) and the mediation of rapid drug tolerance in *Drosophila* (2, 22, 23). Clinically relevant concentrations of ethanol (10–100 mM) can potentiate (20, 24–26) or inhibit (27–29) the activity of BK_{Ca} channels, depending on the origin of the channel. In addition, in supraoptic neurons, ethanol increases nerve terminal BK_{Ca} channel activity but fails to modulate cell body BK_{Ca} channels (26, 30). Efforts are underway to determine the basis of this selectivity, concentrating on different isoforms of the channel-forming (slo) α -subunit (28, 31, 32) and its combination with different β_{1-4} accessory subunits (26).

Although the focus of the actions of ethanol is now on proteins, membrane lipids play an important role in ethanol modulation of ion channel function. Of particular interest, in the case of BK_{Ca} channels, are findings that BK_{Ca} channels as well as other voltage-gated K⁺ channels are preferentially targeted to certain lipid domains (lipid rafts) that are enriched in sphingomyelin and cholesterol (33–35). The activity of these BK_{Ca} channels is influenced by the cholesterol/phospholipid ratio (36) and lipid composition in the surrounding lipid bilayer (37–39). Ethanol activation of BK_{Ca} channels is mediated by the cholesterol content in lipid bilayers (13), and certain structural features of phospholipids (such as molecular shape) may influence the ethanol sensitivity (40).

In a previous study (3), we found that ethanol tolerance in the BK_{Ca} channel in terminals of central nervous system neurons has

two components: a decreased ethanol potentiation of channel activity occurring within minutes of ethanol application, and a slower form of tolerance that results in a decreased number of channels in terminal membranes after 24 h of chronic ethanol treatment. Here, we use a combination of molecular biology and planar bilayer single channel recording techniques to address a previously unexplored aspect of alcohol tolerance, asking whether a single channel protein complex (BK_{Ca}) incorporated into a simple bilayer can exhibit tolerance (*i.e.* is tolerance intrinsic to the protein complex?). We follow the affirmative answer to this question, obtained in a POPE²/POPS bilayer, with a study of the influence of a range of lipid bilayer compositions that manipulate bilayer thickness, on the initial and sustained response to ethanol. This particular manipulation was chosen to capitalize on the findings from a recent series of studies that provide a model to explain the effect of bilayer thickness on BK_{Ca} function (41), and the fact that BK_{Ca} channels are reported to be localized within comparatively thick lipid rafts in the membrane (33–35). To systematically alter bilayer thickness, the BK_{Ca} (*hSlo* α -subunit) channels were reconstituted into lipid bilayers made of DOPE with phosphatidylcholines (PCs) of different acyl chain length from C14:1 to C24:1, and DOPE with brain sphingomyelin (SPM) (41). We found that BK_{Ca} channels reconstituted into thick SPM membranes showed a different response to acute ethanol exposure when compared with the same channel reconstituted into thin bilayers. This indicates that lipid environment surrounding the protein plays an important role in modulating ethanol action on the protein.

In each of these experiments we always have at least two major components, the protein and the lipid bilayer. Because there are a wide variety of lipid compositions in which the BK_{Ca} protein responds similarly to alcohol application (21) we tend to favor the notion that interaction with the protein is the most likely site of action. Although the alternate interpretation, in which the lipid component is the primary site of action, cannot be ruled out, we assume that the lipid composition modulates the response of the protein.

EXPERIMENTAL PROCEDURES

Materials

DOPE, brain SPM, and PCs (from C14:1, to C24:1) were obtained from Avanti Polar Lipids (Alabaster, AL). They were used without further purification. Decane and salts were from Aldrich. Ethanol (100%, anhydrous) was purchased from American Bioanalytical (Natick, MA). All aqueous solutions were prepared with 18.3 m Ω /cm Milli-Q water (Millipore Corp., Billerica, MA).

Membrane Preparation

The cDNA encoding *hSlo*, kindly provided by Dr. P. Ahring, NeuroSearch A/S (Copenhagen, Denmark), was overexpressed in HEK-293 cells. Stably transfected HEK-293 cells were grown in artificial medium (42) and membrane fragments were pre-

pared using a protocol developed for COS cells with some slight modifications as described elsewhere (13).

Electrophysiology

HEK-293 Cell Recordings—HEK-293 cells stably expressing *hSlo* α channels were cultured in 35-mm culture dishes until 45–60% confluence was reached. Cells were washed for 30 min in high calcium (2.2 μ M) bath solution followed by a 10-min wash in intracellular recording solution (1 μ M calcium) prior to recording. Single-channel recordings were performed in excised inside-out membrane patches using standard patch clamp techniques (43). All recordings were made under symmetric K⁺ conditions where the potassium concentration was the same in the bath and recording solutions. Electrodes were fabricated from glass pipettes (Drummond Scientific, Broomall, PA), pulled using a Model P-97 Brown/Flaming micropipette puller (Sutter Instrument, Novato, CA), and coated with sylgard (Dow Corning, Midland, MI) to reduce capacitance and noise. The tips were fire polished using a microforge (Narishige, Kyoto, Japan) to yield electrodes with resistances between 7 and 15 m Ω when filled with high K⁺ extracellular recording solution. An agar bridge containing an Ag/AgCl pellet and 3% agar in buffer solution was used as a ground. Single-channel currents were recorded using an EPC-9 (HEKA Elektronik, Lambrecht, Germany) patch clamp amplifier at a bandwidth of 3 kHz and were low-pass filtered at 1 kHz using an 8-pole Bessel filter (model 902LPF, Frequency Devices, Haverhill, MA) and sampled at 5 kHz using PULSE software (44). Data were acquired and stored using an A/D converter and a Dell computer. Single-channel conductances were obtained from *I/V* plots. Each patch was recorded at a given voltage from –80 to +60 for 5 s.

Solutions—The high-calcium bath solution contained (in mM) 135 Na⁺ gluconate, 5 K⁺ gluconate, 2.2 CaCl₂, 1 MgCl₂, and 15 HEPES. The high-K⁺ extracellular recording solution contained (in mM) 140 K⁺ gluconate, 2.2 CaCl₂, 4 EGTA, 4 HEDTA, 1 MgCl₂, and 15 HEPES. The 1 μ M calcium intracellular recording solution contained (in mM) 140 K⁺ gluconate, 5 Na⁺ gluconate, 0.43 CaCl₂, 2 HEDTA, 1 MgCl₂, and 15 HEPES. Solutions were brought to pH 7.35 with KOH or NaOH as needed. These conditions were optimized for seal formation and patch stability.

Planar Bilayer Recording—Single channel recordings were carried out with standard planar bilayer technology (45). Binary lipid mixtures of DOPE with PCs (1:1, molar ratio) or SPM (3/2, molar ratio) were initially dissolved in chloroform. The solvent was removed by evaporation with a N₂ stream and then the dried lipid film was resuspended in decane to form a final total lipid concentration of 25 mg/ml. The bilayer was formed by painting the lipid solution across a 250- μ m aperture in a Delrin bilayer chamber (model CD-P250 from Warner Instruments, Hamden, CT). Bilayer capacitance was monitored by noting the current across the bilayer in response to a triangle wave (10 mV/25 ms). Membrane suspensions containing crude membrane fragments (0.2–0.5 μ l) were directed to the bilayer in the *cis* chamber with a micropipette. The cytoplasmic *cis* solution contained: 300 mM KCl, 1.03 mM CaCl₂, 1.1 mM HEDTA, 10 mM HEPES, pH 7.2. The free Ca²⁺ was measured with a Ca²⁺ elec-

² The abbreviations used are: POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylserine; DOPE, 1,2-dioleoyl-3-phosphatidylethanolamine; HEDTA, *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid; *P*_o, open probability; PC, phosphatidylcholine; SPM, sphingomyelin.

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trode to be about 20 μM . Ca^{2+} standard solutions were from World Precision Instruments (Sarasota, FL). Additional drops of 0.1 M HEDTA were added to the *cis* chamber to lower free Ca^{2+} concentration so that a low nP_o could be achieved. This allowed us to study ethanol potentiation of BK_{Ca} channels in lipid bilayers of POPE/POPS and SPM/DOPE. The recording solutions were modified from those used in the patch experiments to obtain stable bilayer recordings and the free Ca^{2+} concentrations were chosen to get a modest channel open probability (P_o). Alcohol was added as pure ethanol to the *cis* buffer in amounts necessary to reach the desired 50 mM concentration. Vigorous mixing of the buffer solutions was achieved by continuous stirring of both chambers with a stir bar at its full power (Sun Stir3 from Warner Instruments). The extracellular (*trans*) solution in the inner chamber contained: 150 mM KCl, 0.1 mM HEDTA, 10 mM HEPES, pH 7.2. Single channel currents were recorded with a patch clamp amplifier (EPC-9, HEKA Elektronik, Lambrecht, Germany) (44). The *trans* chamber was connected to ground and all voltages in the *cis* chamber was expressed relative to ground. The holding potential was usually at 20 mV unless stated otherwise. At least 1 min of recording was taken as a control after insertion to ensure stable channel activity before application of ethanol. Continuous recordings were taken after the application of ethanol to obtain the time course of the ethanol response. All experiments were done at room temperature (22 °C).

Ethanol Analysis

To evaluate potential changes in ethanol concentration during the recordings, 100- μl aliquots of buffer were removed at 1, 4, 7, and 10 min after application. Ethanol concentration was determined with the alcohol dehydrogenase assay following the manufacturer's recommendations (Sigma). Briefly, NAD-alcohol dehydrogenase (Sigma) was re-suspended in 50 mM sodium pyrophosphate buffer (Sigma) and aliquoted. Samples of recording buffer solution, along with ethanol standard dilutions, were added to the buffer, incubated for 30 min in room temperature and the absorbance was measured at 340-nm. Calculation of ethanol concentrations was performed according to the manufacturer's formula using the dilution curve of known ethanol concentrations.

Data Analysis

nP_o was calculated as an index of steady-state channel activity from the all points histogram and the number of channels (n) in the recording and the open channel probability (P_o) as described elsewhere (24). Data are expressed as mean \pm S.E. All analyses were done with TAC and TAC-fit programs (Bruyton Corp., Seattle, WA).

RESULTS

Recordings of BK_{Ca} channel activity were obtained in both membrane patches pulled from transfected HEK cells, and after their extraction and incorporation into an artificial bilayer. Fig. 1 illustrates the experimental paradigm.

BK_{Ca} Channels Expressed in HEK Cells Develop Acute Tolerance to Ethanol—The identity of stably expressed BK_{Ca} channels was confirmed by examining the single channel currents

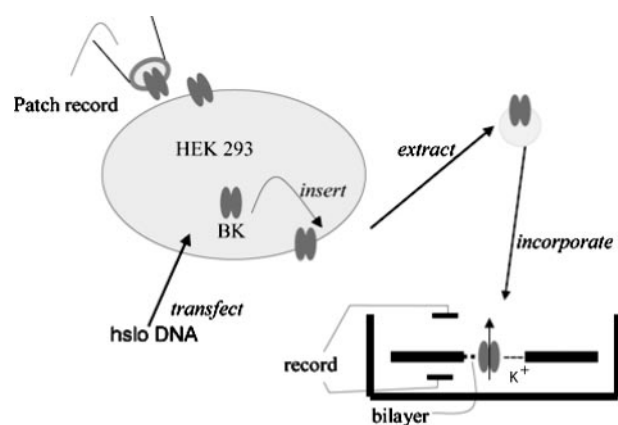


FIGURE 1. A schematic of the experimental strategy employed to examine the BK_{Ca} channel in a natural lipid environment and in an artificial membrane. Stably transfected HEK-293 cells were grown in artificial medium (42) and then half of the cells were used directly in the patch recording experiments. The other half were used to isolate membrane fragments for the bilayer recordings.

recorded in inside-out membrane patches, using symmetric 140 mM K⁺ solutions (Fig. 2). First, the single channel current observed was voltage dependent at a fixed internal free- Ca^{2+} concentration (1 μM). Channel activity increased with depolarization (Fig. 2A). Second, I-V plots of single channel current *versus* membrane holding potential yield a large average unitary conductance of 267 ± 14 pS ($n = 10$) (Fig. 2B). And finally, at a given holding potential, the nP_o increases as the internal free- Ca^{2+} concentration is increased (data not shown). These properties are all consistent with the presence of the expressed BK_{Ca} channel.

Our experimental design provides the ability to monitor the BK_{Ca} channel response to alcohol in a native excised membrane patch and again, after being reconstituted in an artificial bilayer (46). The responses to ethanol in an excised inside-out patch from a transfected HEK cell are shown in Fig. 3.

These patches are thought to retain a relatively complex membrane composition and architecture, as well as any associated cytoskeletal and sub-bilayer structures. Patches were first exposed to the intracellular recording buffer solution ($[\text{Ca}^{2+}]_{\text{free}} = 1 \mu\text{M}$) without ethanol for 3 min to determine a stable control value for steady-state channel activity nP_o (pre-ethanol). Then the patch was exposed to the buffer solution with 50 mM ethanol for 15 min. On average, acute exposure of the cytosolic side of I/O patches to 50 mM ethanol increased channel activity (nP_o) to a maximum potentiation of $\sim 300\%$ in 2 to 3 min after ethanol exposure. This activation gradually relaxed and channel activity returned to pre-ethanol levels within 15 min (Fig. 3B) in the continued presence of the drug (*closed symbols*). In control recordings (*open symbols*), channel activity remained relatively constant indicating that the time course of activation and tolerance seen with the drug is a function of the response of the protein complex to alcohol.

BK_{Ca} Channels Reconstituted in POPE/POPS Bilayers Exhibit Acute Tolerance—To examine whether the BK_{Ca} channel displays acute tolerance in artificial membranes, BK_{Ca} channels extracted from transfected HEK cells were reconstituted into POPE/POPS bilayers. POPE/POPS forms a negatively charged lipid bilayer and has been used in previous studies (13,

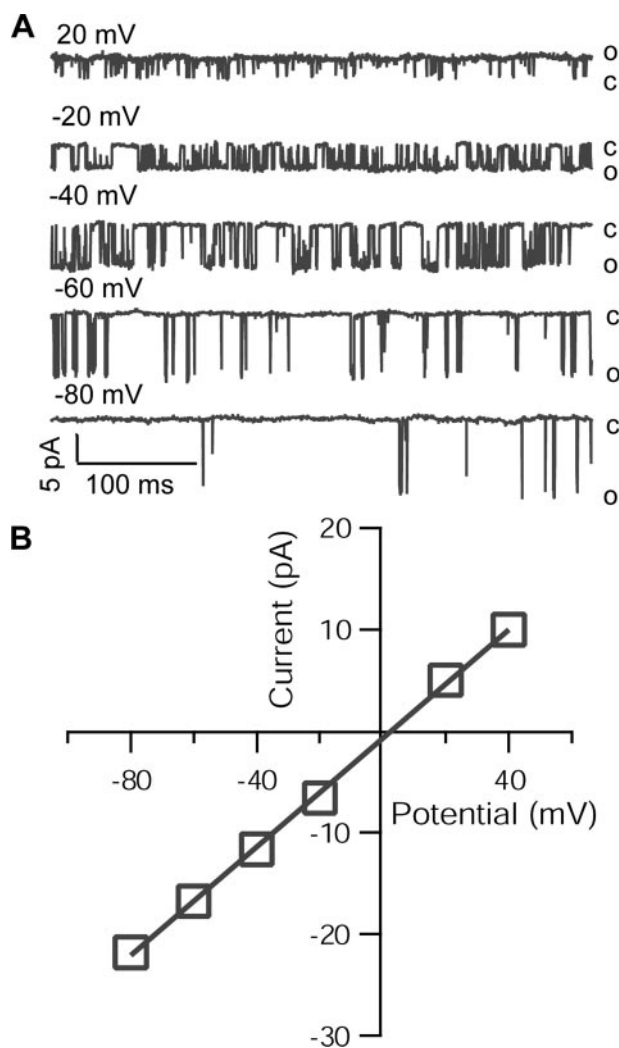


FIGURE 2. Voltage dependence of BK_{Ca} channels expressed in HEK-293 cells. *A*, currents through a single channel recorded in inside-out patch clamp mode in the presence of 1 μ M free Ca²⁺, shows an increase in activity with depolarization from a holding potential of -80 to $+20$ mV. *B*, an I-V plot (channel current versus membrane potential) averaged over 10 recordings yielded a BK_{Ca} channel unitary conductance of 267 pS. *O* and *C* represent the open and closed states of the channel, respectively.

14, 40). The extracted channel protein has been shown to shed native lipids, and to be dominated by the composition of the artificial bilayer lipids into which it is incorporated (13, 14, 40). The BK_{Ca} channels recorded in POPE/POPS bilayers showed an average conductance (γ) of 335 ± 4 pS ($n = 6$) and an average single channel P_o of 0.637 ± 0.078 ($n = 6$), in recordings with asymmetric KCl concentrations (300/150 mM, *cis/trans*); 20 μ M Ca²⁺_{free}, and a holding potential of $+20$ mV.

To observe activation by ethanol, and to avoid a potential ceiling effect, the initial channel activity was reduced by decreasing the free Ca²⁺ to a final concentration of 5–8 μ M by the addition of 0.1 mM HEDTA (40). The addition of ethanol (to a final concentration of 50 mM) quickly increased the channel steady-state activity (nP_o) in POPE/POPS bilayers (see Fig. 4A), as was seen earlier (13, 40). The time course of the ethanol response showed, on average, a peak in 2 to 3 min after exposure to ethanol. The nP_o ratio (peak ethanol/control response) indicated about 300% potentiation (see Fig. 4B, *closed symbols*).

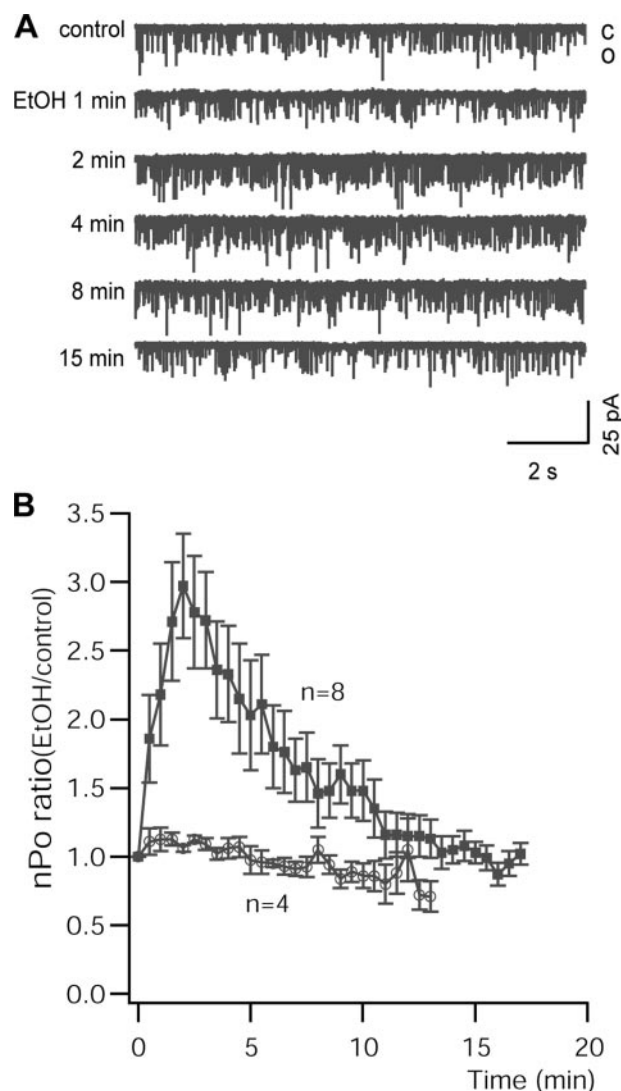


FIGURE 3. BK_{Ca} channels expressed in HEK-293 cells develop rapid acute tolerance to ethanol exposures. *A*, continuous recording of an inside-out patch before and after application of 50 mM ethanol. The holding potential is -50 mV. The traces shown were taken during the first 10 s of 1-min segments of the recording at 1, 2, 4, 8, and 15 min after the application of 50 mM ethanol, as indicated. *B*, normalized plot of nP_o ratio (with ethanol/control) over the time course of recording from 8 patches. Each data point is the nP_o calculated during 30 s of the recording.

Channel activity then rapidly diminished to only a 30–40% potentiation after 10 min of continuous exposure. Control experiments without the addition of ethanol indicated that fluctuations in channel activity were well below 30% of baseline activity over this duration (Fig. 4B, *open symbols*).

Ethanol is a volatile agent and can pass through lipid bilayers within seconds (47). To examine if the reduced potentiation of BK_{Ca} shown in Fig. 4B resulted from a decrease in ethanol concentration during the recording period, the concentration of ethanol in the buffer solution was monitored by taking 0.1-ml samples from the *cis* and *trans* chambers at 1, 4, 7, and 10 min after the application of 50 mM ethanol to the *cis* chamber. The sample solutions were measured with an alcohol dehydrogenase assay (see the *inset* in Fig. 4B). The ethanol concentration in the *cis* chamber remained relatively unchanged, suggesting that the amount of ethanol incorporated, evaporating, or

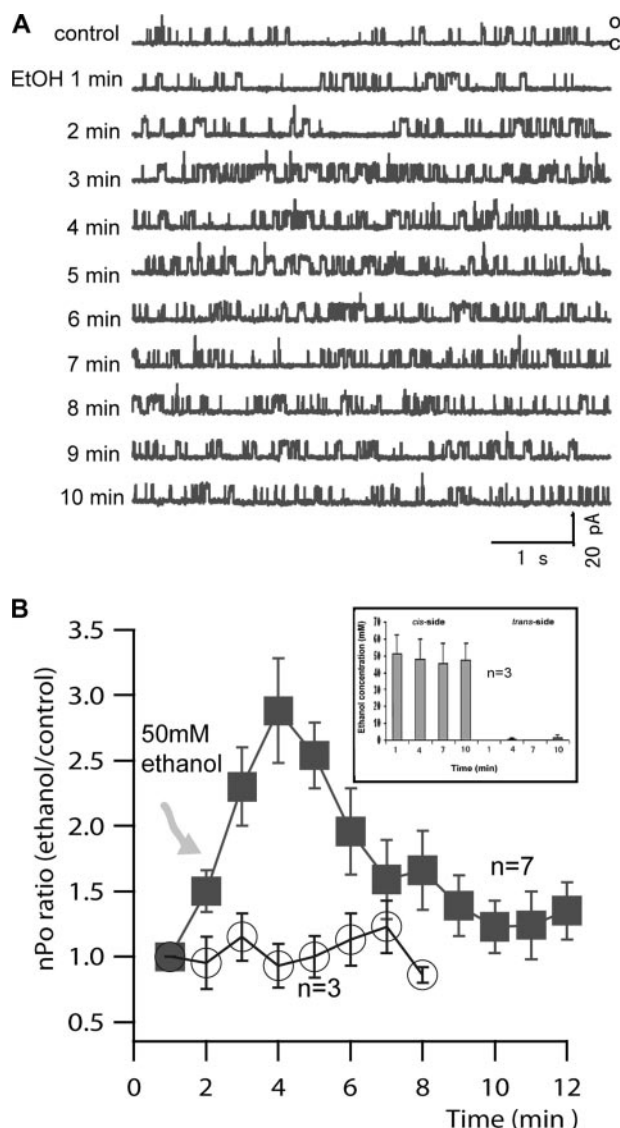


FIGURE 4. BK_{Ca} channels extracted from transfected HEK-293 cells and reconstituted into POPE/POPS bilayers show acute tolerance. *A*, continuous recording of a BK_{Ca} channel in a POPE/POPS bilayer before and 1 min after the application of 50 mM ethanol (indicated by the arrow; buffer conditions: 300/150 KCl (*cis/trans*), [Ca²⁺]_{free} about 8 μM). The holding potential is 20 mV. The traces shown were taken during the first 10 s of each 1-min recording, as marked in the figure. *B*, normalized plot of *nP_o* ratio (with ethanol/control) from 7 trials in POPE/POPS bilayers (filled symbols). Three control experiments (open symbols) showed that BK_{Ca} channel activities are stable over the time course of recording in the absence of alcohol. The inset in *B* shows the measured ethanol concentration in the *cis/trans* recording chambers during a recording time of 10 min.

passing through the lipid bilayer during the recording is negligible. Therefore, the reduced potentiation of the BK_{Ca} channel activity in POPE/POPS bilayers is not due to changes in ethanol concentration, but rather reflects a property of the protein in response to alcohol, similar to that observed in the patch recording.

Membrane Lipids Affect the Appearance of Acute Ethanol Tolerance in the BK_{Ca} Channel—At this point we know that BK_{Ca} channels in both patches and bilayers show patterns of response to alcohol that fit an accepted definition of acute molecular tolerance but we do not have an understanding of the molecular mechanisms that produce this pattern. We

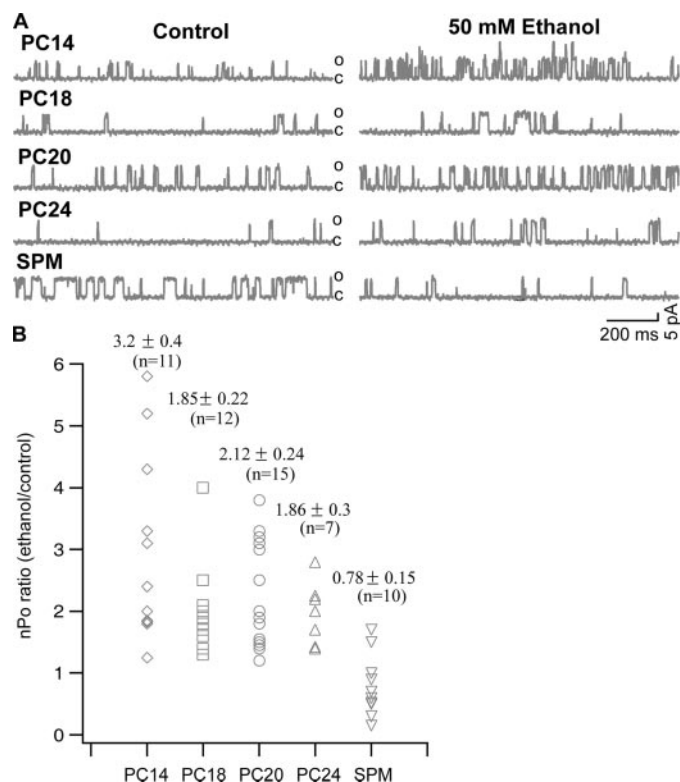


FIGURE 5. Initial ethanol sensitivity of the BK_{Ca} channel changes with an increase in bilayer thickness from robust activation in bilayers of PC (14:1)/DOPE to inhibition in bilayers of SPM/DOPE. *A*, recordings of BK_{Ca} current in each bilayer before and after the application of 50 mM ethanol (2 min after the exposure to ethanol). The buffer condition is 300/150 KCl (*cis/trans*), and 20 μM [Ca²⁺]_{free} for bilayers of DOPE with PC series, and 10 μM for SPM/DOPE bilayers. The holding potential is 20 mV for all bilayers. *B*, scatter plots of BK_{Ca} channel response at 2 min after exposure to 50 mM ethanol in each bilayer. Each point represents a single experiment in a given bilayer. The average *nP_o* (with ethanol/control) are shown as mean ± S.E. at the top of each column, along with the number of experiments in the average.

attempted to gain some insight into these mechanisms by reconstituting the channel into lipid bilayers of different thickness, each made from equal molar DOPE with monounsaturated PCs with chain lengths of C(14:1) to C(24:1) and 3:2 (molar ratio) DOPE/SPM. We had previously confirmed that these compositions produce bilayers of increasing thickness with x-ray diffraction and atomic force microscopy techniques (41).

The initial response to ethanol was monitored 2 min after exposure and expressed as the *nP_o* ratio (ethanol/control). The 2-min time point was chosen partly because BK_{Ca} channels in the membrane patch and in the POPE/POPS bilayer showed maximal ethanol potentiation near this time, and partly because, for some bilayers, such as PC (14:1)/DOPE and PC (18:1)/DOPE, which are not extremely stable, it is difficult to maintain the bilayer for much longer recording periods. Fig. 5*A* shows typical recordings in each bilayer before and after a 2-min application of 50 mM ethanol. Ethanol activates (with a *nP_o* ratio > 1.3) the BK_{Ca} channel in most of these bilayers (10 of 11 for PC (14:1)/DOPE bilayers; 9 of 12 for PC (18:1)/DOPE bilayers; 11 of 15 for PC (20:1)/DOPE bilayers; and 7 of 7 for PC (24:1)/DOPE bilayers). However, in SPM/DOPE bilayers, ethanol inhibits BK_{Ca} channel activity in 6 of 10 bilayers, with 2 bilayers showing slight potentiation and 2 bilayers showing lit-

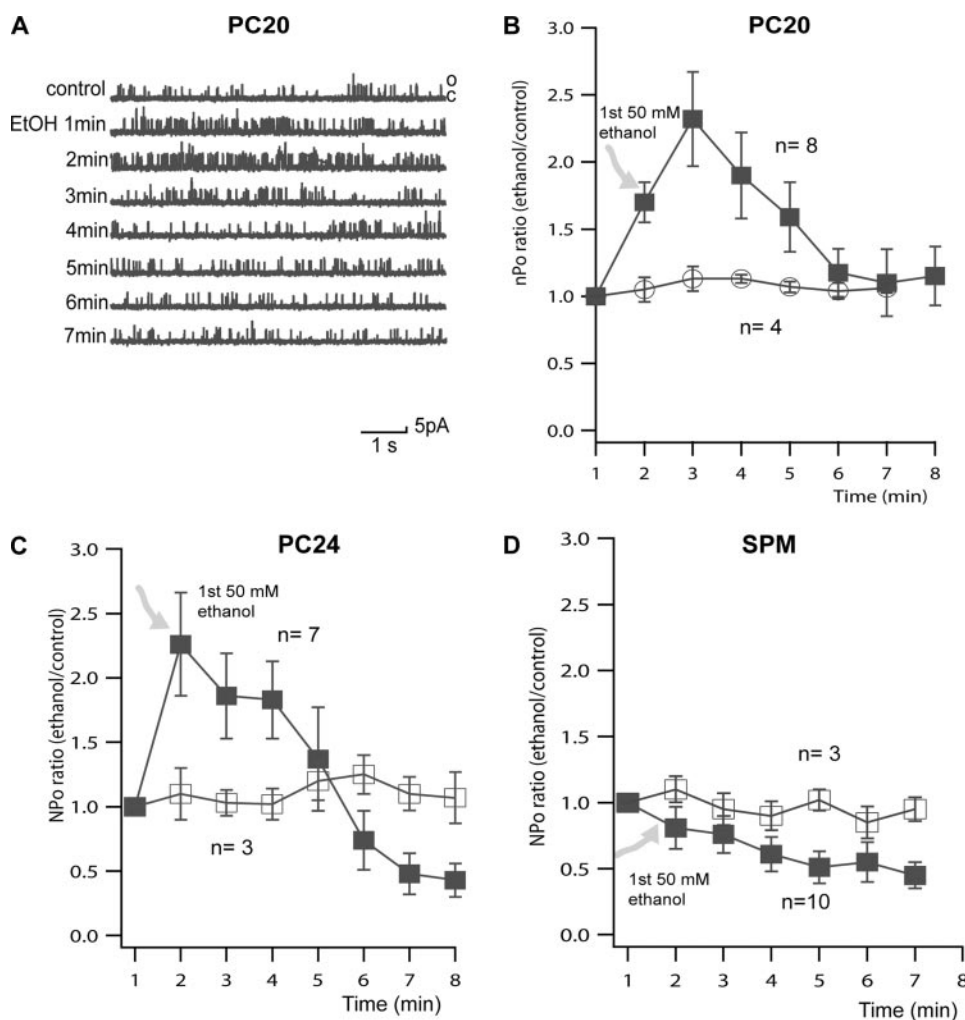


FIGURE 6. The sustained response of BK_{Ca} channels to ethanol is altered in lipid bilayers of different thickness. *A*, traces of BK_{Ca} current from a continuous recording in a PC (20:1)/DOPE bilayer before and after the indicated times since exposures to 50 mM ethanol, showing the development of in session acute ethanol tolerance. The buffer condition is 300/150 KCl (*cis/trans*), and 20 μ M [Ca²⁺]_{free}, holding potential is 20 mV. Normalized plots of *nP_o* ratio (with ethanol/control, closed symbols) over the time course of recording in PC (20:1)/DOPE bilayer (*B*), PC (24:1)/DOPE bilayer (*C*), and SPM/DOPE bilayer (*D*). In each case the open symbols plot the activity in the absence of alcohol and the arrows indicate the first response 1 min after alcohol addition.

tle effect (see Fig. 5*B*). The responses (*nP_o* ratio ethanol/control) ranged from robust activation in a thin bilayer (3.4 ± 0.6 -fold in PC (14:1)/DOPE), to moderate activation (about 2-fold) in PC (18:1)/DOPE, PC (20:1)/DOPE, and PC (24:1)/DOPE bilayers and finally, to inhibition in thick SPM/DOPE bilayers (0.78 ± 0.15). Clearly, with manipulation of bilayer thickness, the early ethanol response of the BK_{Ca} channel exhibits two patterns, potentiation in the thinner bilayers and inhibition in the thicker bilayers.

It is not difficult to envision how these two components if arranged in proper temporal sequence could give rise to a pattern of response that fits the definition of acute tolerance. Indeed, a clearer picture of this possibility emerges when we plot the ethanol response during longer periods of continuous exposure in bilayers of PC (20:1)/DOPE, PC (24:1)/DOPE, and SPM/DOPE, which have enough stability to allow for longer recording periods (Fig. 6). In each case the arrow in the figure indicates the first data point 1 min following the application of alcohol. An example of the traces obtained using this protocol is

shown for a PC (20:1)/DOPE bilayer in Fig. 6*A*. The initial exposure to 50 mM ethanol increased channel activity, reaching a maximum potentiation within 2 min. On average this activation relaxes back to baseline in about 6 min (Fig. 6*B*). This figure also shows that under ethanol-free conditions, BK_{Ca} channel activity fluctuates less than 20% of baseline during the whole of the recording period (Fig. 6*B*, open symbols). Thus, as in POPE/POPS bilayers, in-session acute tolerance is apparent in PC (20:1)/DOPE bilayers, within 6 min of exposure. A similar pattern, potentiation followed by diminution of potentiation within ~5–6 min, was observed in the PC (24:1)/DOPE bilayer (Fig. 6*C*). However, in this case, there was not only a simple reduction in potentiation, but there was an actual reversal of the sign of action of alcohol on channel activity during the longer exposure period, with inhibition of activity to below pre-drug levels evident by the end of the exposure interval. This trend toward inhibition is further evident in the thicker SPM/DOPE bilayer, where ethanol potentiation of channel activity is totally absent, and instead, we observe only inhibition of channel activity. Thus, bilayer thickness appears capable of teasing apart two separate targets of the action of alcohol, which together have the potential to produce the

appearance of acute tolerance. Interestingly, channels incorporated into C-24 bilayers are transitional, exhibiting both initial potentiation and later inhibition. This suggests that the interaction of alcohol with these two processes will prove to have different time courses.

DISCUSSION

The data we present here show clearly that the acute tolerance of the BK_{Ca} channel to ethanol exposure that has been seen in the terminal of central nervous system neurons (3) can be reproduced in the HEK-293 cell, and more importantly, can be retained in the BK_{Ca} channel that we extracted from HEK-293 cells and reconstituted into artificial lipid bilayers. This suggests, given the differences in lipid composition, that the acute tolerance of the BK_{Ca} channel to ethanol exposure is intrinsic to the protein and closely associated entities (including potential channel subunits and modulatory proteins such as phosphatases and kinases that might accompany the channel) (48, 49). Furthermore, we demonstrate that altering the thick-

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ness of the lipid bilayer surrounding the protein modulates the ethanol sensitivity and the appearance of acute ethanol tolerance in the channel.

Acute Alcohol Tolerance Is Intrinsic to the BK_{Ca} Channel Protein—The response of BK_{Ca} channels in intact neuronal terminals to continuous ethanol exposure is characterized by an initial potentiation that wanes within minutes of exposure (3). We began this study asking whether this pattern of molecular tolerance could be maintained as we reduced the preparation first to a membrane patch in another cell type, and then to an isolated channel protein and its tightly bound auxiliary proteins (48) incorporated into a simple POPE/POPS bilayer. That is, is acute BK_{Ca} tolerance intrinsic to the protein, or does it require particular lipid compositions, complex membrane structures, or connection to cytoplasmic elements? Our answer to this question was that tolerance was, indeed intrinsic, because the pattern of tolerance was qualitatively similar in channel recordings from the intact terminal, from the channel studied in the excised HEK cell patch, and from the same channel studied in the POPE/POPS bilayer.

Alcohol tolerance is generally divided into rapid acute tolerance (minutes to hours) and slow chronic tolerance (hours to days) (50). BK_{Ca} channels (3) as well as many receptors, such as *N*-methyl-D-aspartate and GABA_A receptors (50, 51) show fast adaptation to ethanol and have been implicated in adaptive changes in synaptic function in response to ethanol. The data we present show that BK_{Ca} channels exposed to ethanol in excised patches and reconstituted in POPE/POPS bilayers were quickly activated and that the activation gradually diminished during continued ethanol exposure (in about 15 min for patches, and about 6–8 min for bilayers). The rapidity of the fast change in ethanol sensitivity seems to preclude an effect of ethanol at the transcriptional and translational levels, but retains the possibility of second messenger modulation (such as phosphorylation or dephosphorylation) (50) of the channel in the adaptive response (32). The fact that in such a simplified system, extra energy resources are absent in the recording solution, makes phosphorylation less likely, but leaves dephosphorylation as a reasonable possibility because the isolated BK_{Ca} channel has been shown to retain an association with auxiliary proteins such as kinases and phosphatases (48).

Membrane Lipids Play an Important Role in Modulating the Ethanol Action on the BK_{Ca} Channel—As we expanded the study to a series of bilayers designed to vary in thickness, it became clear that bilayer properties significantly influence the response of BK_{Ca} channels to ethanol. Previous work showed that manipulation of bilayer thickness has profound effects on intrinsic biophysical parameters of BK_{Ca} channel function such as conductance and gating (41, 52). Here, we find that bilayer thickness also has powerful effects on the actions of ethanol on BK_{Ca}, changing the sign of action of ethanol from channel potentiation to channel inhibition as the thickness of the bilayer is increased. An important consequence of these findings is that protein and associated lipid should be treated as an interacting system rather than as independent entities when studying the molecular underpinnings of ethanol action.

BK_{Ca} channels are potentiated by clinically relevant concentrations of ethanol (10–100 mM) from many tissues, such as

neurons and neuroendocrine cells (20, 24–26). However, the response is not universally, potentiation. For example, BK_{Ca} channels from vascular tissue are primarily inhibited by ethanol (27). This inhibitory response was ascribed to the presence of the β_1 accessory subunit (27). In addition, in supraoptic neurons, ethanol potentiates nerve terminal BK_{Ca} channel activity but fails to modulate cell body BK_{Ca} channels (26, 30). Because lipid composition is highly specific for membranes from different tissues, and from different parts of individual neurons (53), lipid environment could play a major modulatory role in the different responses seen with exposure. In fact, in addition to thickness, we have seen that changes in the cholesterol concentration in the membrane can effectively antagonize ethanol action on the BK_{Ca} channel (13).

Our results also suggest the possibility that the acute tolerance observed in the membrane patch and in the POPE/POPS bilayer is the combined result of two separate processes (potentiation and inhibition) elicited by alcohol. These are teased apart by manipulating bilayer thickness. We have previously determined, using a combination of atomic force microscopy and x-ray diffraction, that the thickness of bilayers studied here range from 56 Å (d-space) for PC 14:1 membranes to 71 Å for SPM membranes (41). The two patterns of response are especially apparent in the C-24 bilayer, in which both potentiation and inhibition are clearly evident, in a time-dependent manner. The combination of potentiation and inhibition, given the appropriate temporal and amplitude characteristics, could lead to the pattern of acute tolerance seen in any neuronal BK_{Ca} channel.

The apparent fractionation of the ascending and descending limb of the potentiation response we observed by substituting other bilayers that vary in thickness can be explained by a number of alternative scenarios. These include a single site of action for alcohol on the BK_{Ca} channel, with bilayer thickness modulating drug binding and the consequences of drug binding to this site. Alternatively, we can envision two sites on the protein, one a potentiating site that is quickly activated, and a second, inhibitory site, less quickly activated. One analogy for the two-site hypothesis comes from work on the β_2 -adrenergic receptor (54), in which low (nanomolar) concentrations of isoproterenol, a β -adrenergic agonist, caused decreases in the sensitivity of the cellular adenylyl cyclase response to the agonist, without changing the maximal responsiveness, whereas exposure to high (micromolar) concentrations of isoproterenol resulted in decreases in both sensitivity and the maximal responsiveness to agonist. In that study, exposure of cells to low concentrations of agonist preferentially induced phosphorylation at protein kinase A sites. This phosphorylation correlated with the decreased sensitivity to agonist stimulation of an adenylyl cyclase response. At higher agonist concentrations phosphorylation on both the β_2 -adrenergic receptor kinase and protein kinase A sites occurred, and only then was the maximal cyclase responsiveness elicited by agonist reduced. In the two-site scenario, some bilayers would have both sites exposed, whereas others of different thickness could act to selectively expose one or the other site, or could precipitate conformational changes in the BK_{Ca} protein, such that binding of alcohol to one of the two sites is more or less effective. Support for a two-site model

comes from a study in *C. elegans* that has identified two residues in the slo protein that modify channel response to ethanol and conditions ethanol-induced motor incoordination (1). One (cslo, Ile-1001) is located in the S10 segment and the second (cslo, Lys-350) is located in S6.

A mechanistic understanding of action of alcohol is hindered by many unknowns, including the physical basis of: (a) modulation of channel function by bilayer composition, (b) interaction of ethanol with and modulation of the channel protein, and (c) actions of ethanol on the bilayer. Thus, a discussion of mechanism is necessarily highly speculative. To understand how the thickness of the bilayer might affect the action of ethanol on the BK_{Ca} channel, we need to consider the interaction of ethanol not just with the protein as discussed above but also with the lipid bilayer. NMR studies have shown that ethanol binds to the membrane lipid interfacial region, but not to the hydrocarbon interior of the bilayer. It also significantly disorders the entire acyl chain (55). As in the model previously developed to explain the relationship between bilayer thickness and BK_{Ca} channel gating (41), if alcohol reduced the ordering in thin lipid bilayers it would activate the channel. On the other hand, increasing bilayer thickness either by an increase in acyl chain length or incorporation of cholesterol into lipid membranes enhances bilayer ordering, which might counter the ethanol disordering effect on the membrane, leading to reduced ethanol activation of the BK_{Ca} channel (13). In addition, it has been reported that incorporation of cholesterol into lipid bilayers result in a decrease in ethanol binding to the interfacial region of the bilayer (56). So in our two-site model we must include the possibility that one of the sites of action is on the protein and the other is on or in the lipid bilayer.

Membrane lateral heterogeneity is now accepted as a requirement for the function of biological membranes. Cellular membranes contain lipid domains (lipid rafts) that are enriched in sphingomyelin and cholesterol and have different thickness (57) compared with the rest of membrane. Together with our increasing appreciation of ion channel structure and function (58), recent advances in the study of cell membrane organization are changing to reflect the broader functional role of the plasma membrane. The lipid bilayer not only provides a matrix for the membrane protein but also actively participates in signal transduction (by interacting with the protein) (41).

At the moment, we do not have sufficient data to allow us to discriminate among the various mechanistic models including the one-site and two-site possibilities. Clearly, we must also include the possibility of direct interactions between the drug and the lipids surrounding the channel protein, in addition to multiple direct interactions between alcohol and the protein. However, we can say with confidence that the intrinsic response of the channel complex to alcohol is more complicated than originally envisioned.

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