ACCELERATED COMMUNICATION

Distinct Structural Features of Phospholipids Differentially Determine Ethanol Sensitivity and Basal Function of BK Channels

John J. Crowley, Steven N. Treistman, and Alejandro M. Dopico

Program in Neuroscience, Departments of Neurobiology (J.J.C., S.N.T.) and Psychiatry (S.N.T.), and Brudnick Neuropsychiatric Research Institute (S.N.T.), University of Massachusetts Medical School, Worcester, Massachusetts; and Department of Pharmacology, the University of Tennessee Health Science Center, Memphis, Tennessee (A.M.D.)

ABSTRACT

Large conductance Ca^{2+}-activated K^+ (BK) channel activity and its potentiation by ethanol are both critically modulated by bilayer phosphatidylserine (PS), a phospholipid involved in membrane-bound signaling. Whether PS is uniquely required for ethanol to modify channel activity is unknown. Furthermore, the structural determinants in membrane phospholipid molecules that control alcohol action remain to be elucidated. We addressed these questions by reconstituting BK channels from human brain (hslo) into bilayers that contained phospholipids differing in headgroup size, charge, and acyl chain saturation. Data demonstrate that ethanol potentiation of hslo channels is blunted by conical phospholipids but favored by cylindrical phospholipids, independently of phospholipid charge. As found with ethanol action, basal channel activity is higher in bilayers containing cylindrical phospholipids. Basal activity and its ethanol potentiation in bilayers containing phosphatidylcholine, however, are not as robust as in those containing PS. These results are best interpreted as resulting from the relief of bilayer stress caused by inclusion of cylindrical phospholipids, with this relief being synergistically evoked by molecular shape and negative headgroup charge. Present findings suggest that hslo gating structures targeted by ethanol are accessible to sense changes in bilayer stress. In contrast, hslo unitary conductance is significantly higher in bilayers that contain negatively charged phospholipids independently of molecular shape, a result that is likely to be dependent on an interaction between anionic phospholipids and deep channel residues coupled to the selectivity filter.

Large conductance, Ca^{2+}-activated K^+ (BK) channels play a pivotal role in both the behavioral response to acute EtOH exposure (Davies et al., 2003) and the cell adaptations that accompany protracted drug administration (Knott et al., 2002). BK channel activity in neurons and neuroendocrine cell types is usually potentiated by EtOH (Dopico et al., 1996,

1999; Jakab et al., 1997; Knott et al., 2002; Martin et al., 2004). However, EtOH potentiation of BK channels is not universal. In hormone-releasing supraoptic neurons, nerve terminal BK channels are potentiated by EtOH, whereas cell body BK channels are refractory to similar concentrations of the drug (Dopico et al., 1999). Furthermore, BK channels from vascular tissues are primarily inhibited by EtOH (Walters et al., 2000; Liu et al., 2003, 2004b). Experimental conditions make it unlikely that the reported differences in EtOH action on BK channels can be explained by differential modulation of drug action by cytosolic messengers. Differences in EtOH action on BK channels across different tissues and cell domains may be orchestrated by a variety of molecular mechanisms, including expression of different isoforms

ABBREVIATIONS: BK channel, large conductance Ca^{2+}-activated K^+ channel; POPE, 1-palmitoyl-2-oleoyl-phosphatidylethanolamine; POPS, 1-palmitoyl-2-oleoyl-phosphatidylserine; PS, phosphatidylserine; HEDTA, *N*-(2-hydroxyethyl)ethylene-diaminetriacetic acid; DOPE, 1,2-dioleoyl-phosphatidylethanolamine; DOPS, 1,2-dioleoyl-phosphatidylserine; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl-phosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

of the channel-forming (slo) subunit (Liu et al., 2003), channel accessory β_4 subunits (Martin et al., 2004), modulation by membrane-bound signaling molecules, and regulation by the lipid matrix in which the channel protein complex is imbedded.

Protracted EtOH exposure is known to alter the lipid composition of neuronal plasma membranes (Taraschi et al., 1991), which, in turn, may provide a mechanism of tolerance to channels that are sensitive to the lipid environment, such as BK channels (Chang et al., 1995; Crowley et al., 2003; Park et al., 2003). It is interesting that native BK channels display tolerance to protracted EtOH exposure, which results in reduced inhibition of neuropeptide release by alcohol (Knott et al., 2002). Moreover, we demonstrated that BK channel-forming subunits cloned from human brain (*hslo*) exhibit EtOH-induced potentiation after channel reconstitution into 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE)/1-palmitoyl-2-oleoyl-phosphatidylserine (POPS) [3:1 (w/w)] bilayers; this potentiation is largely blunted when POPS is removed from the lipid bilayer (Crowley et al., 2003). The molecular mechanisms underlying the key role of PS in controlling EtOH action on ion channel function are unknown.

Phosphatidylserine is a critical phospholipid constituent of natural membranes. It is particularly abundant in the inner leaflet of the cell membrane and serves as an anchor for membrane-associated signaling molecules that regulate ion channel activity. For example, PS is involved in Ca^{2+}-dependent protein kinase C translocation to the membrane, this kinase being a well known modulator of both basal activity (Schubert and Nelson, 2001) and EtOH potentiation of BK channels (Jakab et al., 1997). Thus, it is conceivable that the presence of PS in cell membranes is specifically required for EtOH to modulate BK channel function given the links that exist between this phospholipid and key cell signaling molecules. On the other hand, the critical role of PS in EtOH modulation of channel function may result from a requirement for a lipid microenvironment with distinct physical properties, which one or more structural features of the PS molecule (headgroup size and charge, overall molecule shape) fulfills. In this case, other naturally occurring membrane phospholipids that also fulfill this requirement will be able to sustain alcohol action independently of cell signaling molecules specifically linked to PS.

In this study, we examined the structural determinants underlying modulation of alcohol action on BK channel function by PS and other relevant naturally occurring membrane phospholipids. We addressed whether PS is specifically required for EtOH to modulate BK channel function and which structural properties of PS and related phospholipid species are required for alcohol action. Addressing these questions allowed us to consider specific bilayer properties as determinants of EtOH action on BK channels. Furthermore, evaluation of single channel function in a variety of artificial planar phospholipid bilayers before and after EtOH exposure allowed us to dissect common and distinct bilayer-mediated mechanisms that influence different aspects of channel function, such as ion conduction, gating properties and EtOH sensitivity.

Materials and Methods

Human Embryonic Kidney 293 Membrane Preparation. 293 cells stably transfected with *hslo* cDNA (provided by Dr. P. Ahring, NeuroSearch A/S, Ballerup, Denmark) were grown to confluence, pelleted, and resuspended on ice in 10 ml of buffer: 30 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, and 5 mM EGTA, pH 7.2. A membrane preparation was obtained using a sucrose density gradient from a cell suspension as described elsewhere (Crowley et al., 2003). Aliquots were stored at $-80°C$ until use.

Electrophysiology. Channels were incorporated by dropping 0.5 to 1 μl of the membrane preparation onto preformed bilayers composed of differing phospholipid mixtures. Lipids were dried under nitrogen gas and resuspended in decane at a concentration of 25 mg/ml. Bilayers were formed by painting the lipid mixture across a 100-μm hole formed in a plastic coverslip (Crowley et al., 2003). Capacitance was monitored by the capacitative current generated with a triangle pulse (20 mV/25 ms). Vesicle fusion was promoted by an osmotic gradient, with the *cis* chamber (to which the vesicles were added) hyperosmotic to the *trans*. Only channels with Ca^{2+} sensors facing the *cis* chamber were studied.

Recording solutions consisted of: *cis*, 300 mM KCl, 10 mM HEPES, 1.10 mM HEDTA, 1.05 mM $CaCl_2$ ($[Ca^{2+}]_{free} \cong 50 \mu M$), pH 7.2; *trans*, 30 mM KCl, 10 mM HEPES, and 0.1 mM HEDTA, pH 7.2. Basal channel function was studied at 50 μM free $[Ca^{2+}]$. Ethanol sensitivity was tested at 10 μM free $[Ca^{2+}]$, which yields a lower channel activity from which EtOH potentiation can be determined. Free $[Ca^{2+}]$ in the *cis* chamber was adjusted using aliquots from a 1 M stock solution of HEDTA, pH 7.2. Free $[Ca^{2+}]$ values given are nominal, calculated using the Max Chelator Sliders program (C. Patton, Stanford University). Because EtOH activation of slo is independent of voltage across the membrane within the range studied here (Dopico et al., 1996), data obtained at *cis* chamber potentials between -10 and $+60$ mV were pooled.

Experiments were performed at room temperature. Single channel events were recorded at a bandwidth of 10 kHz using a patch clamp amplifier (model 8900; Dagan Corp., Minneapolis, MN) and stored on videotape using pulse code modulation (model DMP-100; Nakamichi, Tokyo, Japan). Data were low-pass–filtered at 1 kHz for display and analysis using an eight-pole Bessel filter (model 902; Frequency Devices, Haverhill, MA) and digitized at 10 kHz.

Data Analysis. Data were acquired and analyzed using pClamp (5.5.1 and 6.0.2, Axon Instruments, Union City, CA). As an index of steady-state channel activity, we used the product of the number of channels in the bilayer during recording (N) and the open channel probability (P_o), calculated as described elsewhere (Dopico et al., 1996). Only bilayers containing a single channel ($N = 1$) were used for experiments measuring differences in basal channel P_o. Unitary slope conductance values were obtained by linear fit of plots of unitary current amplitude (i) versus membrane holding potential (V). Linear fits with $r^2 < 0.95$ were excluded from the analysis. When testing alcohol sensitivity, N was monitored before and after EtOH administration by stepping to positive potentials to maximize P_o. Experiments showing an increase in N after EtOH additions were discarded. $(N)P_o$ was determined from periods of at least 20 s of continuous recording.

Data are shown as mean $\pm$ S.E.M. The significance of the difference between means was determined by analysis of variance and tests *a posteriori* (Bonferroni's multiple comparison test).

Chemicals. All solutions were prepared with Milli-Q water and ultrapure grade salts. Ethanol (100%, anhydrous) was purchased from American Bioanalytical (Natick, MA), decane (>99% pure, anhydrous) from Sigma-Aldrich (Milwaukee, WI), and POPE, POPS, 1,2-dioleoyl-phosphatidylethanolamine (DOPE), 1,2-dioleoyl-phosphatidylserine (DOPS), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) from Avanti Polar Lipids (Alabaster, AL).

Results and Discussion

Phospholipid Headgroup Size Is a Critical Molecular Determinant of Hslo Channel Activation by Ethanol. Hslo channels were reconstituted into POPE/POPS (3:1) bilayers. This bilayer composition was chosen to facilitate the comparison of data obtained in the present study with previous results evaluating alcohol action on native BK channels (Chu et al., 1998) and hslo channels (Crowley et al., 2003) reconstituted into this binary bilayer. Exposure of hslo channels to clinically relevant concentrations of EtOH (50 mM) typically resulted in a robust increase in channel steady-state activity (NP_o) (Fig. 1A). This representative result was observed in 82% (9 of 11) of bilayers; NP_o ratios in the presence and absence of EtOH averaged 4.8 ± 1.1 (Fig. 2, first column). In sharp contrast, EtOH failed to increase hslo channel activity in 9 of 11 pure POPE bilayers; NP_o ratios in the presence and absence of EtOH averaged 1.47 ± 0.6 (Figs. 1B and 2, second column). We have shown previously that hslo channel refractoriness to EtOH activation in POPE bilayers remained even when the [EtOH] was raised to 100 mM (Crowley et al., 2003). This [EtOH] maximally activates hslo and other BK channels in natural membranes (Dopico et al., 1996; 1999). Together, our results suggest that POPS is critical for ethanol to activate BK channels.

To determine whether 1) the disparity in EtOH sensitivity between negatively charged POPE/POPS [3:1 (w/w)] and neutral POPE bilayers results from a specific requirement for PS or 2) other negatively charged phospholipid species could render hslo channels sensitive to EtOH, we tested whether hslo channels reconstituted into POPE/POPG [3:1 (w/w)] bilayers are sensitive to EtOH. Both POPG and POPS contain negatively charged headgroups. The former, however, contains a smaller headgroup, yielding a more conical shape to the molecule, in contrast to the more cylindrical shape of POPS (Lee et al., 1993). The overall shape of a phospholipid, determined by the relative volume of the polar headgroup to the hydrophobic acyl chains, can influence the packing energetics of the bilayer and the activity of embedded membrane proteins (Gruner, 1985; Lundbæk et al., 1996; Stubbs and Slater, 1996), as discussed below.

Representative traces of hslo channel activity before (left) and during (right) application of 50 mM EtOH shows that channels are refractory to drug action in POPE/POPG (3:1) bilayers (Fig. 1C). This result was replicated in five other bilayers. Mild channel activation in response to EtOH was observed in only two bilayers; NP_o ratios in the presence and absence of alcohol averaged 0.94 ± 0.21 ($n = 8$) (Fig. 2, third column). Thus, as reported in neutral POPE bilayers, hslo channels reconstituted into negatively charged POPE/POPG bilayers are usually refractory to EtOH-induced activation. These results indicate that the presence of an anionic phospholipid is not sufficient to support EtOH activation of BK channels. On the other hand, EtOH action was indistinguishable in POPE versus POPE/POPG bilayers; both PG and PE have a small headgroup, as opposed to the bulky headgroup of POPS. Together, these results led us to the following alternative hypotheses: 1) PS is specifically required in the bilayer for EtOH to evoke activation of BK channels and 2) a bulky headgroup in the phospholipid molecule (found in several naturally occurring membrane phospholipids other than PS) is sufficient for EtOH to potentiate BK channel activity.

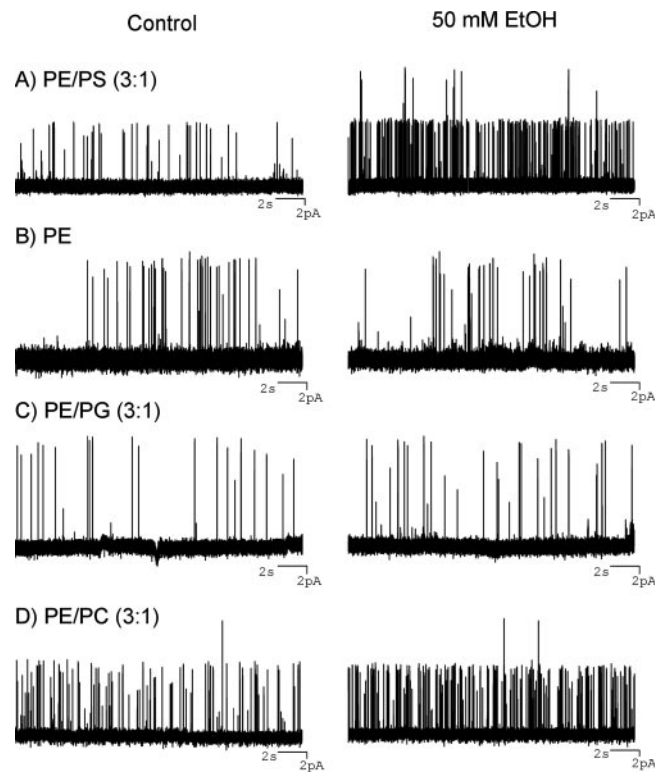To differentiate between these possibilities, we evaluated

Fig. 1. Headgroup size is a key structural feature in membrane phospholipids that determines ethanol action on BK channel activity. Representative traces of the activity of reconstituted BK channels in planar lipid bilayers composed of the indicated phospholipid mixtures before (left) and after (right) exposure to 50 mM EtOH. Traces are representative of experiments in which the [Ca^{2+}]$_{free}$ in the *cis* chamber, to which EtOH was added, was 10 μM, and the membrane was clamped at potentials between -10 and $+60$ mV. Upward deflections in the current trace correspond to channel open events.
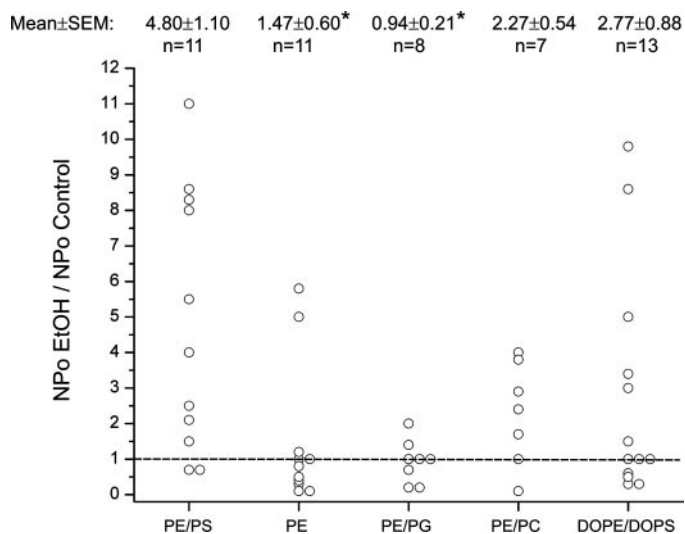

Fig. 2. Phospholipid molecule shape critically modulates ethanol potentiation of BK channels. Summary plot of *hslo* BK channel modulation by 50 mM EtOH. The *y*-axis depicts the magnitude of EtOH action as the ratio of the channel NP_o in 50 mM EtOH to the control period before drug application. Each point represents a single experiment in which the [Ca^{2+}]$_{free}$ in the *cis* chamber was 10 μM, and the membrane was clamped at potentials between -10 and $+60$ mV. The dotted line indicates an NP_o EtOH/NP_o Control ratio of 1, which indicates no change in channel activity. Average NP_o EtOH/NP_o Control ratios are shown as mean $\pm$ S.E.M. at the top of each column of scattered data. *n*, number of bilayers.

EtOH action on hslo channels reconstituted into POPE/POPC (3:1) bilayers. In the presence of POPC, a phospholipid having a bulky headgroup, EtOH typically increased channel NP_o (Fig. 1D). This result was obtained in five of seven bilayers; NP_o ratios in the presence and absence of EtOH averaged 2.27 ± 0.54 (Fig. 2, fourth column). These data indicate that POPS, a key phospholipid species involved in multiple cell signaling pathways (Slater et al., 2002; McLaughlin et al., 2005), is not required in a bilayer or membrane for EtOH to activate BK channels. Rather, the presence of phospholipid species having bulky headgroups, whether zwitterionic (as in POPC) or anionic (as in POPS), seems sufficient.

Overall Phospholipid Molecule Shape Modifies Ethanol Activation of Hslo Channels. Our data demonstrate that phospholipids that have bulky headgroups (POPS and POPC), as opposed to those that have small headgroups (POPG and POPE), markedly favor EtOH activation of hslo channels. These data were obtained with the acyl chain composition of phospholipid species kept constant. This constraint determines that changes in phospholipid headgroup size result in changes in overall molecule shape. It is widely accepted that the overall shape of phospholipid molecules goes from more cylindrical to more conical in the order POPC > POPS > POPG > POPE. Thus, differential EtOH action on hslo channel activity in POPS or POPC versus POPG or POPE containing bilayers could be attributed to dependence of drug action on phospholipid headgroup size per se, overall shape of the phospholipid molecule (e.g., it is possible that the presence of conical phospholipids in the bilayer impedes EtOH activation of hslo channels), or both structural determinants. To explore these possibilities, we modified overall phospholipid molecule shape without altering the headgroup structure itself.

Given a defined headgroup structure, phospholipid molecular shape becomes more conical with an increase in acyl chain unsaturation because of an increase in hydrophobic volume (Gruner, 1985; Lee et al., 1993). Thus, we evaluated EtOH action in bilayers containing two monounsaturated acyl (oleoyl) chains. Figure 3 shows representative traces of hslo channel activity in DOPE/DOPS [3:1 (w/w)] bilayers before (left) and during (right) EtOH exposure. Channels were potentiated by 50 mM EtOH in 6 of 13 bilayers; NP_o ratios in the presence and absence of EtOH averaged 2.77 ± 0.88 (Fig. 2, fifth column). This EtOH-induced potentiation is intermediate, in both frequency and magnitude, between values found in POPE/POPS (EtOH activation in 9 of 11 bilayers; NP_o ratios averaged 4.8 ± 1.1) and POPE/POPG (2 of 8

bilayers; NP_o ratios averaged 0.94 ± 0.21) or POPE (2 of 11 bilayers; NP_o ratios averaged 1.47 ± 0.6). Together, these results demonstrate that, regardless of phospholipid headgroup charge, activation of hslo channels by clinically relevant concentrations of EtOH is significantly reduced by the presence in the bilayer of conical phospholipids, particularly those with small polar headgroups. From our findings, it is not possible to determine the relative contribution of headgroup size and unsaturation of acyl chains in the phospholipid molecule to EtOH action on BK channels. It is clear, however, that conical phospholipid species common in natural membranes impair EtOH activation of hslo channels. Thus, it is conceivable that modification of EtOH action on hslo channel activity by phospholipid headgroup size could represent a class of modulation of EtOH action by the overall shape of the phospholipid molecule.

From a mechanistic standpoint, the differences in EtOH sensitivity between channels in POPE/POPS or POPE/POPC versus POPG or POPE bilayers could result from variations in the energetics of lipid packing, such as the lipid shape-dependent curvature stress within the membrane. The latent energy in the membrane that results from packing conical phospholipids into their less favorable bilayer configuration, termed an elastic curvature stress, is thought to influence the function of several transmembrane and membrane-associated proteins, particularly ion channels (Gruner, 1985; Epand and Bottega, 1988; Mitchell et al., 1996; Stubbs and Slater, 1996). Furthermore, changes in elastic curvature stress represent a key mechanism involved in modification of channel function by interfacially active compounds, including short-chain alkanols (Lundbæk et al., 1996; Cantor, 1999). Curvature stress is antagonized by the addition of more cylindrical lipids, such as POPS or POPC, to the POPE bilayer (Epand and Bottega, 1988). Consistent with the idea that attenuation of curvature stress by cylindrical phospholipids is involved in EtOH potentiation of ion channel activity, EtOH potentiated hslo channels in binary bilayers containing cylindrical phospholipids, independent of phospholipid headgroup charge. This contrasts with the channel refractoriness to alcohol demonstrated in bilayers made of conical phospholipids, such as POPE/POPG (3:1) or pure POPE, where the increase in curvature stress related to these lipids might impair EtOH-induced conformational changes in the channel protein that promote high P_o.

For the majority of ion channel proteins that are sensitive to clinically relevant concentrations of EtOH, amino acid residues that allow the channel to respond to the drug are located in transmembrane positions in the protein (Mihic et al., 1997; Borghese et al., 2003; Sessoms-Sikes et al., 2003). Thus, a reduction in EtOH potentiation of hslo channel activity by conical phospholipids could also be explained by a reduction in EtOH partitioning into the bilayer, limiting access of the drug to a "binding" site located in the transmembrane region of the channel protein. NMR spectra, however, show that EtOH interaction with the lipid-water interface of bilayers cast from *E. coli*-derived PE is similar to that from PC membranes (Barry and Gawrisch, 1994). Because our data show that EtOH action is clearly different in pure PE versus PC-containing bilayers, it is unlikely that a differential partitioning of EtOH across bilayer types can solely account for the differences in the EtOH sensitivity of hslo channels studied in varied phospholipid environments.
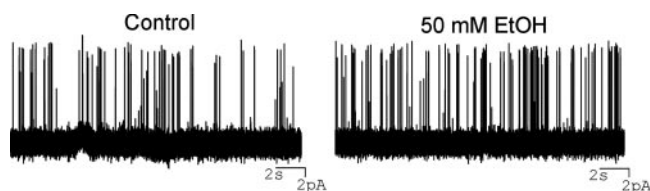

Fig. 3. Ethanol activation of BK channels remains after channel reconstitution into phospholipids having two unsaturated acyl chains. Representative traces of the activity of reconstituted BK channels in planar lipid bilayers composed of DOPE/DOPS (3:1) before (left) and after (right) exposure to 50 mM EtOH. Traces are representative of those obtained with $[Ca^{2+}]_{free}$ in the *cis* chamber at 10 μM, and membrane potentials between -10 and $+60$ mV. Upward deflections in the current trace correspond to channel open events.

By constructing chimeric slo channels, it was recently found that the core domain (S0–S8) of the slo subunit acts as an "EtOH-sensor" (Liu et al., 2003). Furthermore, pinpoint mutagenesis has determined that the presence of a valine in the S0–S1 linker is critical for EtOH to activate slo channels cloned from mouse brain (mslo) (Liu et al., 2004a). Ethanol activation is also observed in slo channels containing an alanine (hslo; from human brain) or a dephosphorylated threonine (bslo; from bovine aorta) in the corresponding position. In contrast, EtOH-activation is significantly impaired by phosphorylation of the threonine in the bslo S0–S1 linker, or mutations of the residue to a negatively charged amino acid (aspartate or glutamate) (Liu et al., 2004a). It is conceivable that the local bilayer properties determined by phospholipid headgroup and charge regulate EtOH binding to this site (characterized by nonstringent amino acid structural requirements) or, more likely, alter the channel protein conformational changes that lead to changes in NP_o after EtOH-binding.

Influence of Bilayer Phospholipid Structure on Basal Steady-State Activity of Hslo Channels. Ethanol-induced increases in BK currents usually occur with minor changes, if any, in unitary conductance. Instead, EtOH action on BK channel function seems to be restricted to an increase in channel P_o, primarily determined by drug-induced destabilization of the channel long-closed state(s) (Dopico et al., 1996; Crowley et al., 2003). Thus, it is possible that the failure of EtOH to potentiate channel steady-state activity in a microenvironment containing conical phospholipids is attributable simply to the fact that the majority of channels already dwell in open states leading to high P_o before EtOH application. Table 1, top row, shows P_o values of hslo channels after reconstitution into lipid bilayers cast from differing phospholipid mixtures under identical recording conditions (0 mV, $[Ca^{2+}]_{free}$ 50 μM, 300 mM/30 mM i/o KCl). Ratios follow the order POPE/POPS (3:1) > DOPE/DOPS (3:1) > POPE/POPG (3:1) ≥ POPE > POPE/POPC (3:1). This order indicates that hslo channels in bilayers containing conical phospholipids do not exhibit higher basal P_o values than those from channels in bilayers containing cylindrical phospholipids. Thus, resistance to EtOH-induced potentiation of channel activity in bilayers containing conical phospholipids cannot be explained simply by the fact that in these bilayers hslo channels dwell in a state(s) of high P_o before drug application.

The activity of a wide variety of transmembrane proteins (e.g., G-protein coupled receptors, ion channels, channel-forming peptides) and membrane-associated proteins (e.g., protein kinase C) are sensitive to shape-dependent differences in lipid packing (Lundbæk et al., 1996; Stubbs and Slater, 1996). As considered above for the EtOH results, the failure of PG to mimic PS modulation of basal P_o could reflect differences in the energetics of lipid packing as a result of the smaller size of the PG headgroup. It is interesting that lyso-phosphatidylcholine, an "inverted cone" phospholipid (which strongly decreases bilayer strain), has recently been found to be a robust activator of native BK channels (Wolfram Kuhlmann et al., 2004). Thus, we may speculate that cylindrical phospholipids lead to increased basal hslo channel P_o because of their promoting positive spontaneous curvature and consequent relief of bilayer stress (see above). Supporting this idea, short-chain alkanols, including EtOH, are thought to favor positive, rather than negative, spontaneous curvature (Chanturiya et al., 1999). Thus, it is conceivable that EtOH potentiation of hslo channels simply mimics cylindrical phospholipid action on channel gating, which is secondary to relief in bilayer stress and/or changes in lateral pressure profile (Cantor, 1999).

A shape-dependent mechanism, however, cannot explain all of our results on phospholipid modulation of basal channel activity. Hslo channels reconstituted into neutral POPE bilayers exhibit P_o values statistically indistinguishable from channels in neutral POPE/POPC (3:1) membranes ($p = 0.145$). This indicates that the presence of cylindrical phospholipids in a zwitterionic bilayer has little influence on hslo channel basal activity. Consistent with previous data obtained with rat brain BK channels (Chang et al., 1995), channel P_o in DOPE/DOPS is not significantly different from that in POPE/POPS, suggesting that alterations in bilayer properties that accompany increased acyl chain unsaturation (among those, overall phospholipid shape) do not critically modify channel steady-state activity. In brief, hslo channel P_o values did not follow the trend predicted were headgroup volume the only determinant of steady-state channel activity. Rather, basal P_o seems to be synergistically potentiated by the combination of large headgroup size/cylindrical shape and negative charge, both of which are structural attributes that are found in naturally occurring PS.

In general, the data in Table 1 indicate that steady-state channel activity is higher in negatively charged (POPE/POPS, DOPE/DOPS, POPE/POPG) than in neutral (POPE/POPC, POPE) bilayers. However, anionic POPG cannot match the enhancement of P_o elicited by addition of POPS to the POPE membrane. Data obtained with rabbit colonic BK channels show that channel activity decreases in PE/phosphatidylinositol (1:1), but not in PE/PS (1:1) bilayers (Turnheim et al., 1999). In addition, the activity of the Na^+/Ca^{2+} exchanger is enhanced by PS, cardiolipin, and phosphatidic acid, but not by phosphatidylinositol or PG (Vemuri and Philipson, 1988). Taken together, our data and these previ-

TABLE 1
Differential modulation of BK channel basal activity and unitary conductance by membrane phospholipids

Summary of basal channel activity in bilayer cast from indicated phospholipid mixtures. All experiments were conducted with the membrane clamped at 0 mV, with 50 μM $[Ca^{2+}]_{free}$ in the *cis* chamber, and a *cis/trans* KCl gradient of 300 mM/30 mM. Only bilayers containing single channels were used to determine the channel steady-state activity (P_o).

	POPE/POPS (n = 25)	POPE (n = 17)	POPE/POPG (n = 21)	POPE/POPC (n = 12)	DOPE/DOPS (n = 9)
P_o	0.544 ± 0.018	0.355 ± 0.027	0.360 ± 0.014	0.236 ± 0.019[a]	0.422 ± 0.028
γ (pS)	337.4 ± 4.3	281.3 ± 11.4[b]	328.5 ± 7.7[c]	304.4 ± 10.1	299.1 ± 11.4[a]

γ, unitary conductance; n, number of bilayers.
[a] Significantly different from values in POPE/POPS ($P < 0.05$).
[b] Significantly different from values in POPE/POPS ($P < 0.001$).
[c] Significantly different from values in POPE ($P < 0.01$) (analysis of variance followed by Bonferroni's multiple comparison test).

ous studies indicate that although naturally occurring anionic phospholipids can modulate the activity of a variety of membrane proteins, the coexistence of negative charge(s) and a bulky headgroup in the phospholipid molecule provides additional subtle tuning leading to increased protein activity.

Phospholipid Negative Charge Is the Primary Determinant of Hslo Channel Unitary Conductance. We next examined whether channel unitary conductance, a parameter of channel function relatively independent of channel gating and unaltered after EtOH exposure of hslo channels (Crowley et al., 2003), could be similarly modified by phospholipid headgroup size and molecular shape. Table 1, bottom row, summarizes the influence of bilayer phospholipid composition on hslo channel unitary conductance. Consistent with reports obtained with other BK channels (Chu et al., 1998; Turnheim et al., 1999; Park et al., 2003), hslo channel unitary conductance was significantly lower in zwitterionic POPE than in negatively charged POPE/POPS ($p < 0.001$). In addition, hslo channels reconstituted into negatively charged POPE/POPG bilayers exhibited unitary conductances similar to those in POPE/POPS ($p = 0.29$), but higher than those in neutral POPE ($p < 0.01$). These data point to a primary role for negative charges in the phospholipid headgroup in promoting high slope conductance values for BK channels, disregarding headgroup size and molecule shape.

Early hypotheses attributed the enhancement in the ion conduction of K^+ channels caused by negative charge in the phospholipid headgroup to an electrostatic attraction of K^+ ions, which increases ion concentration at the mouth of the channel (Turnheim et al., 1999; see Discussion in Park et al., 2003). However, an enhancement of Ba^{2+} block of BK channels in negatively charged membranes, which is predicted by the electrostatic mechanism, is not observed (Park et al., 2003). Rather, the enhanced unitary conductance of the channel may be related to 1) a more direct interaction between the anionic lipid and deep BK channel residues coupled to the channel selectivity filter or 2) differences in the curvature stress associated with phospholipid packing (Park et al., 2003). Our data, however, show that hslo channel conductance in POPE/POPC (3:1) bilayers is statistically indistinguishable from that in pure POPE (Table 1, bottom row). Thus, it is unlikely that elastic curvature stress is a major determinant of hslo channel conductance.

Finally, hslo channel unitary conductance in a bilayer containing only monounsaturated acyl chains, DOPE/DOPS (3:1), is significantly lower (299.1 ± 11.4 pS, $n = 9$; $p < 0.05$) than that in the corresponding mixed chain POPE/POPS (3:1) membrane, suggesting a role for phospholipid molecule shape in regulating channel conductance. Care must be taken, however, in the interpretation of this result, because a variety of physical parameters related to the presence of unsaturated acyl chains might play a role in modulating channel unitary conductance. The rate and range of acyl chain motion ("fluidity") and also the hydrophobic thickness of the bilayer are likely to be different between DOPE/DOPS (3:1) and POPE/POPS (3:1) bilayers (Thurmond et al., 1994). Although a link between membrane fluidity (as evaluated through the mobility of a fluorescence polarization probe) and native BK channel unitary conductance in aortic myocytes could not be detected (Bolotina et al., 1989), bilayer thickness has been causally related to the ion conduction properties of hslo channels (Yuan et al., 2004).

All together, present data highlight the critical role of negative charges on the phospholipid headgroup in regulating BK channel conductance, a modulatory role that is largely independent of the headgroup structure itself. In contrast, phospholipid headgroup shape (headgroup size in particular), but not negative charges, are critical in determining hslo channel gating (see above). This differential modulation of channel conductance versus gating is consistent with structural models of K^+ channels that highlight a deep location in the channel protein for the selectivity filter versus a more accessible to the lipid bilayer location for channel structures involved in gating (MacKinnon, 2003). Given the fact that the pattern in the modification of EtOH action on hslo channel activity is similar to the pattern in the phospholipid modulation of basal channel NP_o, it is possible to speculate that EtOH sensing areas in the BK channel are readily accessible to the lipid bilayer, which seems to be supported by recent mutagenesis studies (Davies et al., 2003; Liu et al., 2004a). In this way, bilayer lipids are likely to influence the channel-EtOH interaction and the transduction of this "binding" event to the channel gate.

Lipids are required for the proper folding, structure, and function of ion channel proteins. We have demonstrated here that ion conduction, steady-state activity, and EtOH sensitivity of hslo channels are all modified by the presence of PS and other relevant membrane phospholipids in the bilayer. However, distinct structural determinants in the phospholipid molecule and underlying bilayer biophysical parameters differentially control these several aspects of membrane channel function. This control might serve as a molecular mechanism to counteract EtOH modulation of channel function and, thus, cell excitability. It has been reported that EtOH administration significantly increases the amount of brain membrane PE (Miller et al., 1997), a conical phospholipid found in our study to oppose alcohol action on channel function.

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Address correspondence to: Alex Dopico, The University of Tennessee Health Science Center, Department of Pharmacology, 874 Union Avenue, Memphis, TN 38163. E-mail: adopico@utmem.edu