Cholesterol and Phospholipid Modulation of BK$_{\text{Ca}}$ Channel Activity and Ethanol Sensitivity: a dissertation

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CHOLESTEROL AND PHOSPHOLIPID MODULATION OF BK_{Ca} CHANNEL
ACTIVITY AND ETHANOL SENSITIVITY

A Dissertation Presented
By
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Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY
In
BIOMEDICAL SCIENCES

June 2003
Cholesterol and phospholipid modulation of $B_{K_{Ca}}$ channel activity and ethanol sensitivity in planar lipid bilayers

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ACKNOWLEDGEMENTS

I would like to thank Steve Treistman for his guidance, insight, and for the opportunity to develop and pursue my research interests in his laboratory. The Treistman lab is a creative, productive, and enjoyable place to work thanks to Steve and the members of his lab (and alumni): Andy, Keith, Paula, Patty, Bob, Bill, Chunbo, Gilles, Sylvie, Andrzej, Nadia, Ben, Tom, and Alex.

I would also like to thank the members of my thesis committee: Tony Carruthers, Josh Singer, Harvey Florman, José Lemos, and Alex Dopico. Their ideas and suggestions greatly improved both the work and the written dissertation. A special thanks to Alex Dopico for the numerous conversations that would start with lipids and channels, but usually end with baseball.

Finally, I would like to thank my parents for their patience and support, and my friends for levity along the way.
ABSTRACT

The large conductance Ca\textsuperscript{++}-activated K\textsuperscript{+} channel (BK\textsubscript{Ca}) regulates neuronal excitability through the efflux of K\textsuperscript{+}, in response to membrane depolarization and increases in intracellular Ca\textsuperscript{++}. The activity of the BK\textsubscript{Ca} channel is increased by acute exposure to ethanol (EtOH), which is thought to underlie, in part, the influence of the drug on peptide hormone release from neurohypophysial nerve terminals (Dopico et al., 1996, 1998). Moreover, chronic EtOH exposure attenuates acute drug action on hormone release, and reduces the sensitivity of BK\textsubscript{Ca} channels to acute EtOH exposure (Knott et al., 2002). The factors regulating EtOH action on BK\textsubscript{Ca} channels are not well understood. Several lines of evidence suggest, however, that the lipid composition of the plasma membrane may influence channel sensitivity to the drug. The plasma membrane is highly complex in its organization (Welti and Glaser, 1994; Brown and London, 1998). There is a growing body of literature indicating that the local lipid composition of the membrane can influence the function of ion channels, including BK\textsubscript{Ca} (Chang et al., 1995a, b; Moczydlowski et al., 1985; Park et al., 2003; Tumheim et al., 1999). Interestingly, chronic exposure to EtOH in animal models results in alterations in the composition of synaptic plasma membranes, including changes in the amount and distribution of membrane cholesterol (CHS) (Chin et al., 1978; Chin et al., 1979; Wood et al., 1989). The significance of these alterations is unclear. Here, we set out to determine the ability of membrane lipids to modulate BK\textsubscript{Ca} channel activity and EtOH sensitivity. To address this, we implement the planar lipid bilayer technique, allowing control of both the protein and lipid components of the membrane. Native BK\textsubscript{Ca} channels
retain EtOH sensitivity in this reductionist preparation (Chu et al., 1998), and we extend the study here to examine cloned human brain \((hslo)\) BK_{Ca} channels.

We show here that \(hslo\) channels maintain their characteristic large conductance, voltage and Ca\(^{++}\)-dependent gating, and sensitivity to 50 mM EtOH in bilayers cast from a 3:1 mixture of 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE) and 1-palmitoyl-2-oleoyl-phosphatidylserine (POPS). The addition of CHS to the bilayer decreases both the basal activity and EtOH sensitivity of the channels, in a concentration-dependent manner. This lends support to the notion that alterations in plasma membrane CHS levels following chronic EtOH exposure may reflect adaptations to the acute actions of the drug on ion channels. Furthermore, the EtOH sensitivity and CHS modulation of these reconstituted \(hslo\) channels are greatly reduced in the absence of negatively charged POPS in the bilayer (pure POPE). Based on these findings, we look to gain mechanistic insight into the lipid headgroup and acyl chain properties that may regulate BK_{Ca} channel modulation by EtOH and CHS. When POPS is replaced with the uncharged lipid 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), the \(hslo\) response to EtOH and CHS is restored, suggesting that the loss of negative surface charge or PS headgroup structure itself cannot explain the lack of channel modulation by these agents in POPE bilayers. Moreover, increases in the proportion of unsaturated acyl chains in the bilayer cannot significantly influence the \(hslo\) response to EtOH. The loss of EtOH sensitivity in pure POPE and CHS-containing bilayers may, therefore, reflect the propensity of POPE and CHS to form nonlamellar (nonbilayer) structures. Regarding the basal activity of the channel, we demonstrate that decreases in negative surface charge, increases in the
proportion of unsaturated acyl chains, and increases in the complexity of headgroup interactions can all influence the steady-state activity of reconstituted \textit{hslo} channels, relative to control POPE/POPS (3:1) bilayers. Overall, these data further suggest the ability of the local lipid environment to regulate the basal function and EtOH sensitivity of an ion channel protein.

Parts of this dissertation have appeared in separate publications:


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INTRODUCTION

The Lipid-based Theory of Ethanol Action on the Nervous System

The molecular mechanisms of ethanol (EtOH) action are not fully understood. The work of Meyer and Overton from the early 20th century demonstrates that the anesthetic potency for a series of structurally diverse molecules, including EtOH, correlates well with their ability to partition into olive oil, a hydrophobic environment. Early reasoning posited that this lipid solubility allows anesthetics to dissolve into the hydrophobic portions of neurons, and alter their activity to produce anesthesia. Though proteins, nucleic acids, and sugars all have hydrophobic regions, the lipids of the neuronal plasma membrane emerged as the most obvious target, especially since the membrane houses the channels and pumps that regulate excitability. EtOH was theorized to partition into and alter the physical properties of this membrane, secondarily affecting the activity of the integral pumps and channels. This “non-specific” mechanism could reconcile the lack of obvious structural similarities in the various anesthetic molecules that would accompany traditional ligand/receptor paradigms. Furthermore, EtOH requires millimolar concentrations for its biological actions, again indicating the lack of a single, high affinity receptor. This general hypothesis guided early research focused on understanding the interaction of EtOH with the lipids that compose biomembranes.

A large body of work employing techniques such as electron paramagnetic resonance (EPR) and fluorescence polarization demonstrates the ability of the small, amphiphillic EtOH molecule to interact with and disorder both native and artificial membranes (Deitrich et al., 1989; Goldstein, 1986). Membrane order refers to the
Figure 1. The influence of ethanol and cholesterol on the order of phospholipid acyl chains.
range of motion (or packing) of the acyl chains of a phospholipid, such that decreases in order correspond to increases in lipid volume. For instance, the introduction of double bonds into phospholipid acyl chains reduces membrane order since they create “kinks” that disrupt the van der Waals interactions between chains that promote tight packing. Both EPR and fluorescence polarization provide a measure of membrane order through the rotational mobility of a probe within the membrane. Tightly packed acyl chains restrict the mobility of the probe, but as the chains become disordered the movement of the probe increases. Data from this sort of experiment demonstrate that 350 mM ethanol can reduce the order parameter of both mouse synaptosomal membranes and artificial vesicles composed of phosphatidylcholine (PC) (Figure 1; Chin and Goldstein, 1981). Furthermore, several studies demonstrate that lipid composition influences EtOH action on the membrane. Cholesterol (CHS), for instance, imparts a concentration dependent increase in the order of PC bilayers (Figure 1). In concurrence, the ability of EtOH to disorder PC vesicles exhibits an inverse correlation to membrane CHS levels (Chin and Goldstein, 1981). Gangliosides, on the other hand, enhance the disordering of both PC bilayers and PC/CHS bilayers by EtOH, when measured using fluorescence polarization (Harris et al., 1984b). These findings suggest that EtOH can alter the physical properties of lipids, and that the properties of certain lipid species may influence the sensitivity of the membrane to disordering by the drug.

A number of studies aimed to correlate the in vivo behavioral response to EtOH in animal models with the propensity for their membranes to be disordered by the drug in vitro. Mice, bred selectively for differences in their sensitivity to the hypnotic effects of
acute EtOH exposure, showed a strong correlation between their behavioral responses and the sensitivity to EtOH disordering exhibited by both their erythrocyte and synaptic plasma membranes (Goldstein et al., 1982). In addition, the synaptic membranes (Chin et al., 1978; Harris et al., 1984a) and erythrocytes (Chin et al., 1978) of mice chronically exposed to EtOH demonstrate tolerance to the disordering effects of the drug *in vitro*. Alterations in lipid composition are thought to underlie this observation, though studies differ widely in the variety of changes reported (Gustavsson, 1990; Swann, 1987; Taraschi et al., 1991).

A major caveat to these studies, however, is the large concentrations of EtOH used to elicit changes in membrane order, usually several hundred millimolar (Chin and Goldstein, 1981; Harris et al., 1984b). Proof of this principle, the importance of membrane order for EtOH action on ion channels, required a demonstration that physiologically relevant concentrations of the drug (22 mM in the blood corresponds to legal intoxication in humans; Diamond, 1992) can influence acyl chain order, and that decreases in order elicit changes in ion channel gating. Ensuing studies provided little data in support of this mechanism. For instance, the magnitude of the lipid disordering caused by anesthetics and n-alkanols is small. These compounds, when tested at concentrations three-fold higher than their respective ED$_{50}$ values for general anesthesia, produce changes in membrane order that are mimicked by temperature changes of less than 1$^{\circ}$ C (Harris and Groh, 1985). Both vigorous exercise and circadian fluctuations alter body temperature by this amount, without leading to anesthesia (Franks and Lieb, 1987). In addition, these compounds do not require lipids to alter the function of certain
enzymes. Firefly luciferase, a soluble cytoplasmic protein, is inhibited by ethanol with an ED$_{50}$ value that correlates well with its respective ED$_{50}$ value for anesthesia (Franks and Lieb, 1984).

The correlation between perturbations of membrane order and ethanol-induced alterations in protein function remains elusive. A large body of literature demonstrates the importance of the physical properties of the membrane in regard to the function of the nicotinic acetylcholine receptor (nAChR) (Barrantes, 1989). The acyl chain order parameter of the native *Torpedo* electroplax membrane, a model for these studies, is 0.8 (arbitrary units). The *Torpedo* nicotinic receptors, when reconstituted into artificial membranes of varying composition, retain functional properties such as agonist binding, gating, and desensitization until the order parameter of the lipid mixture decreases below 0.75 (Fong and McNamee, 1986). The magnitude of this reduction in membrane order is only achieved at excessive concentrations of EtOH, nearing 1.5M. Furthermore, ethanol potentiates agonist-induced currents of the *Torpedo* nAChR, rather than the reduction in activity expected through a reduction in bulk membrane order. It acts with an ED$_{50}$ of approximately 300 mM, far below that presumed necessary to elicit membrane order alterations that impair nAChR function (Miller et al., 1987). Taken together, these results suggest that the mechanism of ethanol action on ion channels is not simply through changes in bulk membrane acyl chain order. In fact, with the advent of cloning, mutagenesis, and expression systems a greater understanding of EtOH interactions with a variety of ion channel classes has emerged. These studies provide the most compelling
evidence for a site of action for EtOH on certain channel proteins themselves, rather than a nonspecific lipid-mediated effect.

**The Shift to Protein-based Theories - Evidence for a Site of Action for EtOH on Ion Channels**

EtOH exposure dramatically alters behavior, and influences the activity of the neurons that underlie it. Early theories suggested EtOH actions on ion channels, the molecules that contribute to neuronal excitability, were transduced through perturbations of the order of the plasma membrane. The EtOH concentrations required to elicit these changes were extremely high, and the degree of change in membrane order quite small and of uncertain significance (Deitrich *et al.*, 1989; Franks and Lieb, 1987). Overall, these observations did not provide a satisfying explanation for the actions of the drug. The advances in cloning and mutagenesis techniques have now allowed researchers to examine the interaction of EtOH with ion channels in tremendous detail. Investigators can compare differences in the EtOH sensitivity across species and subtypes for a given ion channel class. The sequence/domain requirements of these molecules that contribute to regulation by ethanol and other n-alcohols can be assessed through mutation. Data compiled with these powerful approaches, samples of which are discussed below, has solidified the shift from lipid-mediated actions of the drug to more specific protein-based theories of action that currently predominate.

**Glycine and GABA Receptors**

N-alcohols, including EtOH, enhance the agonist-induced currents flowing through GABA_A and glycine (GlyR) receptors. The GABA _p_1 receptor, though, is
inhibited by these agents. Swapping domains of the GlyR and GABA α1 protein, through the construction of chimeric receptors, uncovers a 45 amino acid influencing n-alcohol modulation of the currents. Residues within the second and third transmembrane region of the proteins (S267 and A288 in GlyR, S270 and A291 in GABA_α1) are thought to be essential for some aspect of n-alcohol modulation of these receptors, since mutation of these residues can abolish the sensitivity of the channels to n-alcohols (Mihic _et al._, 1997). The hydrophobic pocket between the TM2 and TM3 regions of these molecules, containing the aforementioned amino acids, may actually provide an n-alcohol binding site. This hypothesis is supported by both chain length cutoff studies and the use of irreversible anesthetic derivatives to modulate channel function. The potency of n-alcohol modulation of many ligand-gated channels increases with the chain length of the alcohol, until a cutoff point is reached. Above the cutoff, further increases in the chain length of the alcohol either inhibit the currents, or fail to potentiate them above the level of the n-1 alcohol. The interpretation of this phenomenon is that there is a binding site of a finite size, into which only alcohols of a certain molecular volume will fit. The cutoff differs widely among the different classes of ligand-gated channels. While S267I mutations in the GlyR abolish sensitivity (Mihic _et al._, 1997), S267Q mutations retain sensitivity but decrease the cutoff from 10 carbons to 3 carbons, suggesting this residue is involved in the binding of n-alcohols (Wick _et al._, 1998). This interpretation was bolstered by a study employing propanethiol (similar to propanol), which can enhance current amplitude reversibly, but can also covalently modify cysteine residues in the presence of iodine (I₂). Wild-type GlyRs are potentiated by propanethiol, but this
enhancement of current can still be washed out even following treatment with I₂. An S267C mutation yields irreversible modulation by propanethiol/I₂, suggesting that covalent linkage to this residue traps the molecule in the n-alcohol binding site. Irreversibly modified channels exhibit a reduced sensitivity to octanol, indicating that modulation is through a single site (Mascia et al., 2000).

Nicotinic Acetylcholine Receptors (nAChR)

The agonist-induced currents of the nicotinic acetylcholine receptor (nAChR), like the GABA and GlyR, are also sensitive to n-alcohols. The receptors are composed of a variety of α and β subunits, that show distinct expression patterns throughout the nervous system. Depending on subunit composition, ethanol can either inhibit or activate these channels. The α4β2 combination, one of the predominant heteromeric receptors in the central nervous system, is potentiated by EtOH (Cardoso et al., 1999). The amino acids in positions homologous to S267 and A288 of GlyR, however, do not appear to constitute an n-alcohol binding site on the α2 subunit of the nAChR (Borghese et al., 2002). Cutoff studies with nAChR reveal further differences from the binding site characteristics of the GlyR. Interestingly, the currents from this channel type are enhanced by ethanol, propanol, and butanol. However, pentanol and longer chain alcohols are inhibitory, again differing from that observed with GlyR (Zuo et al., 2001).

The α7 subunit can form homomeric nAChRs, the predominant α-bungarotoxin receptors in the central nervous system, which are inhibited by EtOH. The NH₂-terminal region of this subunit can impart inhibition by EtOH when fused with the transmembrane and COOH-terminal region of the 5-HT₃ receptor, which is normally potentiated by the
drug (Yu et al., 1996). EtOH also inhibits the nAChRs of the peripheral nervous system. Residues in the pore-lining M2 segment of both the α and β subunits of these receptors are thought to provide hydrophobic pockets that allow open state modulation by n-alcohols. Mutations in this region strongly influence the actions of these drugs (Zhou et al., 2000).

**K⁺ Channels**

Voltage-gated Shaw2 K⁺ channels are inhibited by n-alcohols. Mutagenesis studies demonstrate that the linker between the S4 and S5 regions of the α-subunit is necessary for this inhibition, and it can confer sensitivity to hKv3.4 K⁺ channels that normally do not respond to these agents (Covarrubias et al., 1995; Harris et al., 2000b). Work from our laboratory demonstrates that two clones of the large conductance Ca⁺⁺-activated K⁺ (BKca) channel that exhibit a high degree of homology (>90%), mslo and bslo, respond oppositely to the application of ethanol (Dopico and Treistman, 1996; Dopico, 2003). G-protein coupled inward rectifier K⁺ (GIRK) channels are potentiated by EtOH, while the inwardly rectifying K⁺ (IRK) channels are not. The EtOH sensitivity of chimeric receptors indicated that a 43 amino acid region of the carboxyl terminus of GIRK was important for EtOH modulation of the channel (Lewohl et al., 1999).

From the results described above, it is apparent that n-alcohol modulation of ion channel function exhibits a degree of specificity. These experiments demonstrate not only that certain domains of channel proteins are required for drug action, but that these domains can confer or alter the sensitivity to EtOH when part of chimeric channel constructs. These results suggest that the mechanism of ion channel modulation by n-
alcohols is likely to involve a direct interaction with a hydrophobic pocket on the channel protein itself, rather than a nonspecific disordereding of the membrane lipid.

The Importance of Lipid Environment in Ion Channel Function and Ethanol Sensitivity

Ethanol is a small, amphiphilic molecule that can partition into and disrupt the physical properties of the bilayer. The significance of the drug effects on membrane lipids is unclear, though, due to the high concentrations of EtOH required to produce them (Deitrich et al., 1989; Franks and Lieb, 1987). Its anesthetic actions on the nervous system, at relevant physiological concentrations, are thought to result from drug modulation of several classes of ion channels. Channel modulation by EtOH, in many cases, can be linked to domains or specific residues within the sequence of the protein (Harris et al., 2000a; Lewohl et al., 1999; Mihic et al., 1997; Yu et al., 1996; Zhou et al., 2000). As a result, it is generally accepted that EtOH binds in a hydrophobic pocket(s) on the channel protein to influence gating. There are several compelling reasons, though, to revive an interest in lipid composition as it pertains to channel function and EtOH sensitivity.

The sites on channel proteins deemed important for the actions of the drug are, to date, largely located within transmembrane regions (GlyR and GABA\textsubscript{A}; Mihic et al., 1997) or on intracellular sites (Shaw2; Harris et al., 2000a). Partitioning of the drug into the membrane may, therefore, be a vital step in channel modulation that is sensitive to membrane lipid composition. Recent advances in the understanding of membrane domain organization, and channel distribution within it, also provide cause for interest in
the lipid microenvironment. It is increasingly clear that the plasma membrane is highly heterogeneous, and that domains exist within the membrane that can serve important roles in cell function (Bevers et al., 1999; London, 2002; Welti and Glaser, 1994). Data from both native and artificial membranes, discussed below, demonstrate the complexity of the interactions between lipid molecules, as well as integral proteins, that lead to the formation of these domains. It is of particular interest that ion channels have been shown to differentially associate with certain membrane domains (Bravo-Zehnder et al., 2000; Bruses et al., 2001; Delling et al., 2002; Hill et al., 2002; Martens et al., 2000; Martens et al., 2001; Shlyonsky et al., 2003; Suzuki et al., 2001b), as there is increasing evidence to indicate that the local lipid environment of an ion channel can profoundly influence its activity (Bozina et al., 1989; Chang et al., 1995b; Chang et al., 1995a; Levitan et al., 2000; Lundbaek et al., 1996; Moczydlowski et al., 1985; Park et al., 2003; Turnheim et al., 1999). Finally, there is a large body of literature that demonstrates alterations in the membrane composition following chronic exposure to EtOH (Gustavsson, 1990; Swann, 1987; Taraschi et al., 1991), suggesting the possibility that membrane lipid composition is regulated as part of a compensatory response to the presence of the drug. Currently, however, the role of membrane lipid composition in channel sensitivity to EtOH is largely unexplored. These data and their implications for basal ion channel function and EtOH sensitivity are discussed below, and represent the core of the questions to be posed in this thesis.

**Lipid Domains in Biological Membranes- Their Formation, Relevance to Cell Function, and Ion Channel Behavior**
Plasma Membrane Leaflet Asymmetry

The plasma membrane exhibits a complex organization of lipid species both within and across its leaflets. The maintenance of this organization involves interactions between lipids, between lipid and proteins, and the activity of proteins involved in the translocation of lipid species throughout the cell (Bevers et al., 1999; London, 2002; Welti and Glaser, 1994). Glycolipids, phosphatidylcholine (PC) and sphingomyelin predominate in the extracellular leaflet of the plasma membrane, while primary amine-containing phospholipids such as phosphatidylethanolamine (PE) and phosphatidylserine (PS) are localized to the cytosolic face. This asymmetry is a largely ATP-driven phenomenon, regulated by three major enzyme activities: aminophospholipid translocase (flippase), phospholipid translocase (floppase), and Ca\(^{++}\)-dependent lipid scramblase. Flippase localizes PE and PS to the inner leaflet, hydrolyzing approximately one ATP molecule per lipid translocation (Bevers et al., 1999). The buildup of PS on the inner leaflet may underlie the localization of many regulatory and structural proteins to the inner face of the plasma membrane, such as PKC (Palfrey and Waseem, 1985), annexin (Meers and Mealy, 1993), and spectrin (Manno et al., 2002). High Ca\(^{++}\) concentrations inhibit flippase and activate scramblase, leading to a collapse of membrane asymmetry important for platelet activation and macrophage recognition of apoptotic cells (Bevers et al., 1999). Overexpression of wild-type lipid scramblase in mast cells has an inhibitory effect on exocytotic release elicited by a Ca\(^{++}\) ionophore, with a time lag of approximately 5 minutes. If the plasma membrane lipid asymmetry is disrupted before application of the ionophore the inhibition is immediately apparent, suggesting a role for
lipid asymmetry in some aspect of the exocytotic process in these cells (Kato et al., 2002).

In regard to ion channel function, this lipid asymmetry may have interesting functional implications. PS, which is preferentially transported to the cytosolic leaflet, carries a net negative charge at physiological pH, creating a negative surface potential at the inner face of the membrane. In terms of electrostatics, this results in an accumulation of cations, and corresponding depletion of anions (Eisenberg et al., 1979; McLaughlin et al., 1981). BKCa channels reconstituted into negatively charged planar bilayers (composed of PS, PE/PS, or PE/PI) exhibit higher open probability (Po) and slope conductance (g) values, relative to channels in neutral bilayers (composed of PE, and PE/PC) (Moczydlowski et al., 1985; Park et al., 2003; Turnheim et al., 1999). While cation accumulation may contribute to this phenomenon, it is unlikely to represent the sole mechanism by which negatively charged bilayers enhance these BKCa channel properties (Park et al., 2003).

Lateral Domains- Phosphatidylinositol 4,5-bisphosphate (PIP2)

Within the plane of a leaflet, lipids can associate non-randomly due to their physical properties, or to interactions with membrane proteins (London, 2002; McLaughlin et al., 2002; Welti and Glaser, 1994). The size, half-life, physical properties, and physiological role of these domains are a major focus of membrane biology and biophysics. Phosphatidylinositol 4,5-bisphosphate (PIP2) is a phospholipid that plays an important role in many aspects of cell function. It can be hydrolyzed by phospholipase C (PLC) to produce diacylglycerol, which binds and activates protein kinase C (PKC), and
inositol triphosphate (IP₃), a diffusible second messenger that causes the release of Ca²⁺ from intracellular stores. Phosphoinositide 3-Kinase (PI3K) can phosphorylate PIP₂, creating phosphatidylinositol 3,4,5-triphosphate (PIP₃), which serves as an anchor for a variety of signaling proteins (McLaughlin et al., 2002). A number of domains within signaling and structural proteins have been identified that bind PIP₂ and PIP₃, which contribute to the membrane localization and wide array of physiological processes linked to these molecules (Hurley and Meyer, 2001; Lemmon and Ferguson, 2001; Sato et al., 2001). There is evidence that the lateral organization of these lipids is not random. Investigators have made use of a PIP₂ binding domain called the pleckstrin homology (PH) domain, by fusing it to GFP to create a marker for PIP₂ within the cell. By this method, transient domains of PIP₂ are detected in the budding phagosomes of macrophages (Botelho et al., 2000), and the membrane ruffles of epidermal growth factor (EGF) stimulated HeLa cells (Honda et al., 1999). PIP₂ is also localized exclusively to the plasma membrane of hippocampal neurons, and accumulates to the center of boutons after electrical stimulation of the cell (Micheva et al., 2001). This and other data have lead investigators to suggest a dynamic role for PIP₂ domains in exocytosis (Holz and Axelrod, 2002). The mechanism at hand to concentrate PIP₂ in a given domain is currently unclear. One proposed mechanism involves sequestration by a membrane-associated PIP₂ binding protein called myristoylated alanine-rich C kinase substrate (MARCKS), which contains a cluster of basic residues that may electrostatically sequester the trivalent PIP₂ lipid molecules. PKC phosphorylation of MARCKS releases
the presumed PIP₂ sequestering domain from the membrane, suggesting the potential for local release of sequestered PIP₂ (McLaughlin et al., 2002).

PIP₂ hydrolysis regulates the activity of P/Q- and N-type Ca²⁺ channels (Wu et al., 2002), as well as TRP (Runnels et al., 2002) and BKCa (Liu et al., 2003) channels. PIP₂ itself binds directly to KATP channels to modulate their activity. The Kir6.2 subunit of the channel can associate with membranes in a manner similar to the PH-GFP construct used above, and muscarinic M1-mediated phospholipid depletion can reduce this association (Cukras et al., 2002). PIP₂ is thought to compete directly with ATP for a site on the channel, and prevent the inhibition by the nucleotide (Cukras et al., 2002; MacGregor et al., 2002). The negative charge of the lipid is relevant in this phenomenon, as potency for competition decreases with decreasing negative charge, PIP₃ > PIP₂ = PIP > PI. Phosphatidylserine (PS) is also negatively charged, and can inhibit ATP binding despite differences in lipid headgroup structure (MacGregor et al., 2002). Residues in the carboxyl terminus of the Kir6.2 subunit are thought to mediate this interaction with PIP₂ (Cukras et al., 2002).

Cholesterol and Sphingomyelin-rich Lipid Rafts

Another prominent and more widely studied example of a membrane domain is the cholesterol (CHS) and sphingomyelin (SM)-rich lipid raft structures (Figure 2a). These membrane fragments are distinguished biochemically by their insolubility in non-ionic detergents such as Triton X-100 (at 4°C), and their low density that allows them to float in sucrose gradients. Rafts form by virtue of the physical properties and resulting interactions of raft lipids. Lipids undergo a phase transition (Tₘ) from the gel (Lβ) to
Figure 2. A) Schematic of a cholesterol and sphingomyelin-rich lipid raft domain (shaded). Figure adapted from Pike LJ (2003) J. Lip. Res. 44(4):655-667. SPM, sphingomyelin; Chol, cholesterol; Gang, ganglioside. B) Lipid properties and interactions that underlie raft formation. Figure adapted from Fantini J, Garmy N, Mahfoud R, and Yahi, N (2002) Exp. Rev. Mol. Med. GPLs, glycerophospholipids; Lo, liquid-ordered phase; Lc, liquid-crystalline phase; Lβ, gel phase.
liquid crystalline (Lc) phase (Figure 2b). SM contains long, saturated acyl chains, differing from most biological phospholipids (denoted GPLs in Figure 2b) that are rich in kinked, unsaturated chains. As a result SM molecules undergo the transition from the Lβ to Lc phase at higher temperatures. SM molecules pack tightly in the Lβ phase at 37°C, leading to phase separation from the Lc phase (lower Tm) phospholipids in the membrane. The SM-rich rafts in biomembranes, however, are not in the Lβ phase due to the high concentrations of CHS found in the plasma membrane. The structure of CHS consists of a tetracyclic fused ring skeleton, a hydroxyl group at carbon 3, and a hydrocarbon side chain at carbon 17 (see Figure 6, Chapter I). The hydroxyl group orients the planar, rigid molecule in the membrane, by associating with the aqueous phase along with the polar headgroups of the membrane phospholipids. Increasing amounts of CHS in the bilayer can broaden and eventually abolish the sharp Lβ-Lc phase transition of phospholipid bilayers, imparting the bilayer with properties of both the gel phase (ordered acyl chains) and liquid crystalline phase (lateral mobility). This bilayer phase induced by high CHS concentrations is referred to as the liquid-ordered phase (Lo). CHS packs favorably with the saturated acyl chains of SM, further promoting phase separation and raft formation in sphingolipid/phospholipid mixtures. The association of CHS with SM is theorized to involve hydrogen bonding between the hydroxyl group on CHS and the amide linkage of SM (Ohvo-Rekila et al., 2002). The ability of CHS to promote phase separation in sphingolipid/phospholipid mixtures is thought to underlie the ability of CHS depletion to disrupt lipid raft structures (Brown and London, 1998; London, 2002; Ohvo-Rekila et al., 2002).
The functional role of lipid raft domains in neurons includes neurotrophin signaling, cell adhesion, axon guidance, and synaptic transmission. Several molecules that regulate these neuronal functions exhibit raft association including glycosylphosphatidylinositol (GPI)-anchored and transmembrane receptors, GTPases, Src family tyrosine kinases, syntaxins 1A and 3, neuronal cell adhesion molecules (NCAMs) and associated adaptor proteins from the glutamate receptor interacting protein (GRIP) family (Paratcha and Ibanez, 2002; Tsui-Pierchala et al., 2002). There is also a direct link between ion channel localization, function and raft partitioning. Members of the Shaker-like family of voltage-gated potassium (K⁺) channels differentially associate with these raft structures in mouse L cells, and disruption of rafts through CHS depletion alters channel function. Kv2.1 and Kv1.5 both associate with lipid rafts though only Kv1.5 colocalizes with the scaffolding protein caveolin, indicating it resides in a distinct type of raft domain, termed caveolae. On the other hand, Kv4.2 is consistently found in the detergent soluble fraction of the membrane, indicating that it does not partition into these domains. Disruption of membrane CHS disperses rafts, shifts the V_{1/2} for inactivation 40 mV to the right for Kv2.1, and both the V_{1/2} for activation and inactivation approximately 10 mV to the left for Kv1.5 (Martens et al., 2000; Martens et al., 2001). G-protein-activated inwardly rectifying K⁺ (GIRK) channels are also located in lipid rafts. The targeting of these channels within the cell appears to be determined by the presence or absence of NCAM140 in the raft domain along with the channel. Rafts lacking this NCAM allow transport to the cell surface, while channels in NCAM140-containing domains are retained in an intracellular compartment (Delling et al., 2002). Cloned
human BKCa (hslo) channels expressed in Madin-Darby canine kidney (MDCK) cells are sorted preferentially to the apical membrane of these polarized cells. This localization is independent of N-glycosylation. These channels appear to partition into lipid rafts, again suggesting raft association may influence protein targeting in specialized cells (Bravo-Zehnder et al., 2000). The epithelial sodium channel is present in rafts both intracellularly and on the plasma membrane in A6 cells, but it is unclear how this influences channel sorting or activity (Hill et al., 2002; Shlyonsky et al., 2003). Neurons of the chick ciliary ganglion exhibit the formation of clusters of the α7 nicotinic acetylcholine receptor (nAChR) during synaptogenesis. The maintenance of these clusters requires lipid rafts, as CHS depletion disrupts rafts as well as the channel clusters (Bruses et al., 2001). AMPA receptors, GluR1-4, isolated from rat forebrain neurons are also raft-associated (Suzuki et al., 2001a). A recent study, implementing bovine aortic endothelial cells, has demonstrated an inverse correlation between surface density of Kir 2 channels and membrane CHS content, though raft association was not analyzed directly. Interestingly, membranes enriched in epicholesterol, an optical isomer of CHS, exhibit increases in the surface density of Kir 2 channels, suggesting that direct sterol-protein actions may be involved (Romanenko et al., 2002).

The functional importance of the various lipid domains found within the cell continues to be elucidated, and emerging roles in cell surface targeting and signal transduction are apparent. The formation of rafts is largely dependent upon the physical properties of SPM and CHS, which pack tightly to form liquid-ordered domains that resist detergent solubilization. It is this difference in physical properties from the bulk
lipid that allows the isolation of these domains (Brown and London, 1998; London, 2002; Ohvo-Rekila et al., 2002). However, there is little understanding of how these rafts, due to their physical properties, may regulate the activity of transmembrane proteins. An ordered environment rich in CHS or SPM could feasibly alter the activity of these proteins, particularly ion channels that open and close within the plane of the membrane. There are increasing examples of the modulation of channel function by membrane lipid composition, particularly the vital raft component CHS.

The influence of membrane cholesterol has been studied in regard to the function of a number of ion channel classes. The sterol can alter the properties of the volume-regulated anion current. This current, studied in bovine aortic endothelial cells, is elicited by exposure to an osmotic gradient. Depletion and enrichment of membrane cholesterol enhances and reduces, respectively, the sensitivity of the current to a small osmotic stimulus. Anion selectivity is unchanged, suggesting the conduction pore of the channel is unaffected (Levitan et al., 2000). Manipulation of membrane CHS in IMR32 cells demonstrates that increases in the sterol content selectively shift the inactivation of N-type Ca\(^{++}\) channels toward more positive potentials, without influencing voltage activation (Lundbaek et al., 1996). BK\(_{Ca}\) channels, studied in patches pulled from rabbit aortic smooth muscle cells, exhibit increased P\(_o\) when the cells are treated with mevinolin, an inhibitor of CHS biosynthesis that depletes plasma membrane cholesterol. Conversely, enriching the membrane of these cells with CHS-rich liposomes results in a decrease in P\(_o\) (Bolotina et al., 1989). This result was extended with the study of rat brain BK\(_{Ca}\) channels incorporated into CHS-containing bilayers. As the amount of CHS in the
lipid mixture increases, the mean open time, \( P_o \), and conductance of the channel decrease (Chang et al., 1995b).

**Biophysical Insight Into the Mechanism of Cholesterol Action on Ion Channels**

Mechanistically, CHS actions on ion channels have been interpreted in light of the influence of the sterol on the properties of the bilayer. Changes in \( \text{BK}_{\text{Ca}} \) channel \( P_o \) elicited by depletion and enrichment of membrane CHS correspond with a respective increase and decrease in the rotational diffusion coefficient of DPH, measured by fluorescence polarization. This technique provides a relative measure of membrane order (Bolotina et al., 1989). However, it has been suggested that acyl chain order alone cannot explain steady state changes in channel \( P_o \) (Lundbaek et al., 1996). The measurement of \( \text{BK}_{\text{Ca}} \) channel mean open time \( (t_o) \) over a series of temperatures allowed the construction of an Arrhenius plot, indicating that CHS causes a decrease in the activation energy for the transition from the open to the closed state (Chang et al., 1995b). CHS was theorized to decrease the stability of the open state by altering the physical properties of the bilayer (Chang et al., 1995b). Essentially, if the transition from the closed to open state of the channel is accompanied by a change in volume, a force will be transmitted to the bilayer as the channel opens. The properties of the bilayer will determine, in part, the magnitude of the resulting force deflected back on the channel. The addition of CHS may alter the physical properties of the membrane to destabilize the open state of the \( \text{BK}_{\text{Ca}} \) channel.

For instance, the relative volume of the polar versus non-polar area of a lipid molecule influences its ability to pack efficiently into a planar bilayer. Molecules with
larger non-polar regions (relative to polar regions on the same molecule), like CHS, are referred to as "cone" shaped lipids, and can promote nonbilayer phases (Figure 22). Incorporation of such lipids into the membrane increases the stored energy in the bilayer, which may then influence the stability of certain protein conformational states (Figure 23). A more detailed description of lipid molecular shape and its influence on BKCa channel function is contained within Chapter III of the thesis.

The abundance of CHS in the plasma membrane, its ability to regulate membrane proteins and bilayer physical properties, as well as its role in lipid rafts, suggest it is an important modulator of membrane function. The concept of membrane domains suggests that the location of a channel within the membrane could strongly regulate its function. This is of particular interest when considering chronic models of EtOH exposure. A large body of evidence indicates that changes in membrane lipid composition may occur as part of a compensatory response to the continued presence of the drug (Gustavsson, 1990; Swann, 1987; Taraschi et al., 1991). Changes in membrane CHS, in particular, have been well documented (Chin et al., 1978; Chin et al., 1979; Wood et al., 1989). Given its roles in domain formation, domain maintenance, and ion channel modulation described above, it is tempting to speculate that these alterations in membrane CHS following long term EtOH exposure may indeed be relevant to the physiology of adaptation to the drug. A determination of the functional significance of these changes is a major goal of this thesis.

Membrane Cholesterol and its Modulation by Chronic Ethanol Treatment
Early hypotheses regarding EtOH action on the nervous system contended that the drug elicited non-specific effects on channel gating, through disordering of the bulk lipid of the plasma membrane. This prompted numerous studies designed to correlate behavioral sensitivity to EtOH with the sensitivity of membranes to the disordered actions of the drug. Of particular interest was a comparison of the membranes from animals chronically treated with EtOH to those that were naïve to the drug. These studies detected a number of alterations in membrane composition following chronic EtOH treatment, including changes in CHS (Gustavsson, 1990; Swann, 1987; Taraschi et al., 1991). The functional significance of these findings has yet to be clarified. Given the complexity of membrane organization, the emerging roles for lipid domains in cell function, and the sensitivity of channels to membrane lipid environment, it is a worthwhile task to revisit the importance of these changes.

CHS is a ubiquitous component of mammalian cell membranes. The sterol disorders the acyl chains of gel-phase bilayer and orders the chains in fluid phase membranes, maintaining an optimal fluidity for proper membrane function. Aside from its role in regulating membrane properties, cholesterol serves as a precursor to the synthesis of steroid hormones, bile acids, and vitamin D. The cell, therefore, tightly regulates its metabolism to maintain CHS levels at 20 – 40% of total plasma membrane lipid weight (depending upon the cell type). CHS is obtaining by cells by both biosynthesis and transport from lipoproteins in the plasma, both of which are influenced by feedback regulation at the transcriptional through post-translational levels (Ohvo-Rekila et al., 2002). The enzymes involved in the biosynthetic pathway of cholesterol, as
well as the LDL receptor that regulates CHS uptake from the plasma, are under common transcriptional control. Serum response elements (SREs) in the regulatory regions of these genes allow recognition by transcription factors called serum response element binding proteins (SREBPs). These SREBPs are found primarily in the membranes of the endoplasmic reticulum and nuclear envelope. Decreasing sterol levels cause the NH₂ terminus of the SREBP protein to be cleaved off, after which it enters the nucleus to activate downstream genes (Thewke et al., 2000). The mechanism through which this occurs may involve direct sterol binding/sensing by molecules involved in the proteolytic processing of the SREBP proteins (Brown et al., 2002).

Cholesterol antagonizes the EtOH-induced disordering of phospholipid acyl chains (Chin and Goldstein, 1981). Synaptic membranes from EtOH tolerant mice are resistant to disordering by EtOH and, in some cases, exhibit increases in the amount of membrane CHS. This alteration in membrane lipid composition was theorized to represent a compensatory response to the continued disordering presence of the drug (Chin et al., 1978; Chin et al., 1979; Smith and Gerhart, 1982). However, these increases in the synaptic CHS content were not detected in all strains of mice, only C57BL. Increases in membrane CHS also appear in chronic exposure studies of human (Benedetti et al., 1987) and rat (Kanbak et al., 2001; Lalitha et al., 1989) erythrocytes, rabbit spinal cord (Halat et al., 1988), human hepatic cell line WRL-68 (Gutierrez-Ruiz et al., 1995), HeLa cells (Keegan et al., 1983), rat synaptic membranes (Renau-Piqueras et al., 1987; Zerouga et al., 1991), rat hepatocytes (Smith et al., 1982), and cultured rat cerebellar granule cells (Omodeo-Sale et al., 1995). Some studies, however, did not detect changes
in CHS composition (Swann, 1987). Interestingly, changes in the leaflet distribution of the sterol have also been observed following chronic ethanol treatment, in the absence of overall increases in membrane cholesterol composition. The synaptic membranes of mice chronically treated with EtOH exhibited a 2-fold increase in the CHS content of the outer leaflet of the plasma membrane, relative to control mice (Schroeder et al., 1996; Wood et al., 1989; Wood et al., 1999).

The functional significance of these findings remains unclear. It is now obvious, though, that changes in membrane CHS content and distribution can have profound effects on membrane organization (Brown and London, 1998; London, 2002; Ohvo-Rekila et al., 2002) and the behavior of several classes of ion channels (Bolotina et al., 1989; Chang et al., 1995b; Levitan et al., 2000; Lundbaek et al., 1996), currently deemed the relevant targets of drug action. To address the possibility that alterations in membrane CHS represent a compensatory response to EtOH, it is first necessary to determine the ability of the sterol to alter the interaction of the drug with a relevant target. The BK<sub>Ca</sub> is modulated by both EtOH (Chu et al., 1998; Dopico et al., 1996; Dopico et al., 1998; Dopico et al., 1999; Dopico, 2003; Jakab et al., 1997; Knott et al., 2002; Walters et al., 2000) and membrane composition (Bolotina et al., 1989; Chang et al., 1995b; Chang et al., 1995a; Moczydlowski et al., 1985; Park et al., 2003; Turnheim et al., 1999), making it an ideal model with which to pose these questions.

**The Large Conductance Ca<sup>2+;</sup>-activated K<sup>+</sup> (BK<sub>Ca</sub>) Channel-Basic Channel Properties and Relevance to the Physiology of EtOH**

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The large conductance Ca\(^{++}\)-activated K\(^{+}\) (BK\(_{\text{Ca}}\)) channel is expressed in many tissues throughout the body. The channel opens in response to both membrane depolarization and increases in intracellular [Ca\(^{++}\)]\(_{\text{Free}}\), allowing the efflux of K\(^{+}\) ions from the cell. The channel passes K\(^{+}\) ions through the pore at an extremely high rate (\(g>200\text{pS}\)), while still maintaining selectivity for K\(^{+}\) over other monovalents such as Na\(^{+}\). As a result, BK\(_{\text{Ca}}\) channels exhibit many tissue specific functions. In the nervous system, these channels colocalize with Ca\(^{++}\) channels (Marrion and Tavalin, 1998), modulate action potentials (Poolos and Johnston, 1999), and regulate the release of neurotransmitter (Robitaille and Charlton, 1992). BK\(_{\text{Ca}}\) channel gating is relatively complex. The channel can be activated in the absence of [Ca\(^{++}\)]\(_{\text{Free}}\) (Horrigan and Aldrich, 1999; Horrigan et al., 1999), but the \(V_{1/2}\) for activation shifts to the left with increasing [Ca\(^{++}\)]\(_{\text{Free}}\). Functional BK\(_{\text{Ca}}\) channels consist of a tetramer of \(\alpha\) subunits, with each subunit contributing in a stepwise manner to the cooperative activation of the channel by [Ca\(^{++}\)]\(_{\text{Free}}\) (Niu and Magleby, 2002). The \(\alpha\) subunit, shown in Figure 3, is encoded by the slowpoke (slo) gene, named for a Drosophila mutant, which has been cloned from several species including the fly (Adelman et al., 1992; Atkinson et al., 1991), mouse (Butler et al., 1993), and human (Tseng-Crank et al., 1994). The channel has considerable homology to other voltage gated K\(^{+}\) channels in the S1-S6 region, in that it contains an S4 voltage sensor and a P-loop between S5 and S6. It is unique in the presence of an NH\(_2\)-terminal S0 transmembrane domain, and, as a result, an extracellular NH\(_2\)-terminus. It also contains a large (~850 residues) intracellular C-terminus with a regulator of conductance for K\(^{+}\) (RCK) domain, and an aspartate-rich “Ca\(^{++}\) bowl” in the
Figure 3. Schematic of large conductance Ca\(^{++}\)-activated K\(^{+}\) (BK\(_{Ca}\)) channel α and β subunits. Figure adapted from: Qian X, Nimigean CM, Niu X, Moss BL, and Magleby KL (2002) J. Gen. Phys. 120(6):829-843.
tail region. Mutation of the "Ca++ bowl" can reduce $^{45}\text{Ca}^{++}$-binding activity of the tail region from the *Drosophila (dslo)* clone of the BK$_{Ca}$ channel. This coincides with an $\sim 80$ mV rightward shift in the $V_{1/2}$ for activation of the channel when expressed in HEK293 cells (Bian et al., 2001). The RCK domain contains two additional sites important for divalent cation regulation of channel gating. One, in combination with the "Ca++-bowl", appears sufficient to account for the behavior of the channel over a range of $[\text{Ca}^{++}]_{\text{Free}}$ concentrations, while a second low affinity divalent site contributes the modulation of the channel by millimolar concentrations of Ca$^{++}$ or Mg$^{++}$ (Shi et al., 2002; Xia et al., 2002).

However, recent experiments demonstrate that BK$_{Ca}$ channels lacking the intracellular C-terminus, including the RCK domain and the Ca$^{++}$-bowl, are not only functional, but retain their sensitivity to Ca$^{++}$ (Piskorowski and Aldrich, 2002).

Channel diversity is achieved through splice variations, associated proteins, and the expression of auxiliary $\beta$ subunits (1-4) (Figure 3), which exhibit differences in their expression patterns and in their channel modulation. The bovine slo (bslo) gene, for instance, contains 10 alternative splice sites. In chromaffin cells, stress hormones regulate splicing at a site on the intracellular tail of the channel (Lai and McCobb, 2002). Pituitary ablation causes a sharp decrease in the inclusion of a $\sim 60$ amino acid cysteine-rich insert termed stress axis-regulated exon (STREX) (Xie and McCobb, 1998). This exon allows channels to activate faster with smaller depolarizations, and deactivate more slowly (Saito et al., 1997), thereby altering the firing pattern of the cell (Lovell and McCobb, 2001). The precise mechanism by which stress hormones regulate splicing in chromaffin cells remains unclear, though both depolarization and overexpression of
Ca^{++}/calmodulin-dependent protein kinase (CaMK) repress inclusion of the STREX exon (Xie and Black, 2001). In rat myometrium, a 33 amino acid insert termed splice variant 1 (SV1) controls the cell surface expression of the channel. BK_{Ca} channels containing SV1, which is spliced in between S0 and S1, are retained in the endoplasmic reticulum in a dominant-negative manner (Zarei et al., 2001). From these examples, it is clear that splicing regulates not only channel activity, but also its intracellular trafficking. In *Drosophila*, an associated protein termed *dslo* interacting protein 1 (DSLIP1) also reduces cell surface expression of the channel, though no known mammalian homologue is known (Xia et al., 1998). Another *dslo* interacting protein from *Drosophila*, termed *slo*-binding protein (Slob), decreases cell surface expression and increases the activity of the channels when exogenously applied to ripped-off patches (Schopperle et al., 1998). Both proteins were isolated in yeast two-hybrid screens, and both interact with the C-terminus of the channel (Schopperle et al., 1998; Xia et al., 1998). Slob expression in *Drosophila* appears to be under circadian control (Ceriani et al., 2002). There is evidence that Slob binds the zeta isoform of 14-3-3, suggesting it may be part of a regulatory complex that controls channel function (Zhou et al., 1999). Channel activity is also regulated by both the catalytic subunit of cAMP-dependent protein kinase (PKAc) and Src tyrosine kinase, both of which bind simultaneously and directly to the channel (Wang et al., 1999).

Regulation of BK_{Ca} channel function can also be achieved in a tissue-specific way through the expression of several auxiliary β subunits (Orio et al., 2002). The β subunits consist of two transmembrane domains connected by an extracellular linker.
approximately 120 residues long. Four major classes of β subunit have been cloned from mammals. Co-expression of β1 with the α subunit induces a leftward shift in the \( P_o \)-Voltage curve at a given Ca\(^{++}\) concentration, and slows the activation and deactivation kinetics of the channel (Cox and Aldrich, 2000). It is expressed in predominantly in smooth muscle, and targeted deletion of the gene in mice influences arterial tone and blood pressure, which correlate with decreases in Ca\(^{++}\) sensitivity of the BK\(_{Ca}\) channel (Brenner et al., 2000b). The β2 subunit is expressed predominantly in chromaffin cells and brain, and it produces inactivating BK currents. It accomplishes this with an NH\(_2\)-terminal “ball and chain” mechanism originally described for the Shaker K\(^+\) channel (Wallner et al., 1999; Xia et al., 1999). β3 subunits consist of 4 different splice variants (β3a-d) that are expressed in the testis, spleen and pancreas. Co-expression of β3a-c with the α subunit yields partially inactivating BK\(_{Ca}\) current, while β3d had no apparent effect on channel function (Uebele et al., 2000; Xia et al., 2000). β4 is expressed predominantly in the brain, it reduces the apparent Ca\(^{++}\) sensitivity of the BK\(_{Ca}\) channel, and slows channel activation kinetics (Brenner et al., 2000a; Meera et al., 2000). Interestingly, residues in the extracellular loop of the β4 subunit render the BK\(_{Ca}\) channel resistant to blockade by the peptide blockers charybdotoxin and iberiotoxin (Meera et al., 2000). Through splice variants, cytoplasmic kinases, associated factors, and transmembrane β subunits the single slowpoke gene can produce tremendous diversity in the behavior of BK\(_{Ca}\) channels.

Work from this laboratory has shown that BK\(_{Ca}\) channels are relevant targets of EtOH, during both acute and chronic exposure to the drug. EtOH reversibly potentiates
native BK channels in patches pulled from rat neurohypophysial nerve terminals. This finding suggests that significant cytoskeletal structure and diffusible second messengers are not required for channel modulation by the drug (Dopico et al., 1996). Cloned mslo channels expressed in a non-neuronal background, the Xenopus oocyte, are dose-dependently and reversibly activated by ethanol in the ripped-off patch configuration. The response of the channel to EtOH decreases with increasing intracellular \([\text{Ca}^{++}]_{\text{Free}}\), suggesting the drug may behave as a partial agonist on the channel, \(\text{Ca}^{++}\) being the full agonist (Dopico et al., 1998). It is not currently known how the channel responds to EtOH in solutions lacking \(\text{Ca}^{++}\) altogether. Data presented in Appendix I of this thesis suggests that, for reconstituted channels, an exposure to high \(\text{Ca}^{++}\) levels may promote a robust response to a subsequent application of EtOH. Channels from GH3 pituitary tumor cells respond to EtOH in the outside-out patch configuration, and protein kinase C (PKC) inhibitors block augmentation of channel activity by the drug (Jakab et al., 1997).

Rat skeletal muscle T-tubule BK\(_{\text{Ca}}\) channels incorporated into planar lipid bilayers retain EtOH sensitivity. This reductionist preparation, lacking native lipid and cytoskeletal architecture, still maintain dose-dependent modulation by the drug (Chu et al., 1998). Bovine aortic smooth muscle BK\(_{\text{Ca}}\) channels reconstituted into planar bilayers are inhibited by EtOH (Walters et al., 2000), as they are in more complex backgrounds (Dopico and Treistman, 1996; Dopico, 2003). These studies clearly demonstrate that complex membrane domain formation, cytosolic second messengers, and complex cytoskeletal architecture are not required for the modulation of BK\(_{\text{Ca}}\) channels by ethanol.
Rat magnocellular neurons provide a model to study EtOH modulation of BK$_{Ca}$ channel function as it pertains to cell physiology. The cell bodies of these neurons are in the hypothalamus and they project axons to the posterior pituitary (neurohypophysis), where they release the peptide hormones oxytocin and vasopressin into the bloodstream. These neurons exhibit distinct BK$_{Ca}$ channel subtypes in the soma versus the terminals. Channels from the terminals exhibit a lower Ca$^{2+}$ sensitivity than somatic BK$_{Ca}$ channels, and are resistant to charybdotoxin unlike the somatic channel subtype. These observations are consistent with a differential expression of the auxiliary β4 subunit. Intriguingly, the BK$_{Ca}$ channels of the terminals respond to acute EtOH exposure, whereas the cell body variant is unaffected by the drug (Dopico et al., 1999). It is currently unclear if a differential association with the β4 subunit underlies the differences in the BK$_{Ca}$ channel response to EtOH in the two subcellular compartments. Acute exposure to EtOH blocks neuropeptide release from both the intact neurohypophysis and isolated terminals (Knott et al., 2000; Wang et al., 1991a; Wang et al., 1991b). Peptide release is a Ca$^{2+}$-driven process, and EtOH inhibition of release may result from both the inhibition of Ca$^{2+}$ channels (Wang et al., 1994; Wang et al., 1991a; Wang et al., 1991b) and activation of BK$_{Ca}$ channels (Dopico et al., 1996; Dopico et al., 1999) elicited by the drug in nerve terminals. Long-term exposure to EtOH renders the nerve terminals of the magnocellular neurons resistant to inhibition of peptide release by an acute challenge with the drug (Knott et al., 2000). This tolerance phenomenon is accounted for by compensatory changes in the EtOH sensitivity and current density of both Ca$^{2+}$ and BK$_{Ca}$ channels. The concentration dependence for the EtOH inhibition of L-type Ca$^{2+}$ channels
from the terminals of tolerant rats is shifted to the right, and the terminals exhibit an increase in current density. Concurrently, there is a rightward shift in the EtOH activation of BKCa channels, as well as a decrease in current density (Knott et al., 2002). These alterations in channel function would combine to counteract the acute actions of the drug. The mechanisms underlying these changes in EtOH sensitivity and current density are unknown.

**Evidence for a Potential Role of Membrane Composition in EtOH modulation of BK channels**

EtOH acutely and chronically modulates neuronal BKCa channels (Dopico et al., 1996; Knott et al., 2002). Acute exposure to the drug potentiates channel activity (Chu et al., 1998; Dopico et al., 1996; Dopico et al., 1998), though sensitivity to acute EtOH drops sharply after chronic exposure, along with current density (Knott et al., 2002). The mechanism by which this occurs is unknown. It is tempting to speculate a role for membrane lipid composition in these processes.

Mice chronically exposed to EtOH exhibit alterations in the amount (Chin et al., 1978; Chin et al., 1979; Smith and Gerhart, 1982) or distribution (Schroeder et al., 1996; Wood et al., 1989; Wood et al., 1995) of membrane cholesterol (CHS). CHS and sphingomyelin (SPM)-rich lipid raft structures serve an important functional role in cells, including the trafficking of ion channels (Bruses et al., 2001; Delling et al., 2002), including BK (Bravo-Zehnder et al., 2000). CHS is an integral component of rafts, and alterations in membrane CHS can profoundly influence raft integrity (Brown and London, 1998; London, 2002; Ohvo-Rekila et al., 2002). The decrease in BKCa nerve
terminal current density in chronically exposed rats may be attributed to alterations in the intracellular trafficking of the channel, a process sensitive to the dynamics of cellular CHS. The concentration dependence of BKCa channel potentiation by EtOH is shifted to the right following chronic exposure to the drug. The gating of native BKCa channels derived from both muscle and brain is sensitive to the levels of CHS in the membrane. CHS reduces the activity of channel (Bolotina et al., 1989; Chang et al., 1995b), opposite to the actions of EtOH (Chu et al., 1998; Dopico et al., 1996; Dopico et al., 1998; Jakab et al., 1997). The membrane enrichment of CHS following chronic drug exposure may represent a compensatory response to the acute effects of the drug on channel function. Additionally, the presence of CHS in the membrane may inhibit the response of the BKCa channel to acute EtOH exposure. For instance, CHS can antagonize the effect of halothane on nAChR from Xenopus myocytes. Halothane reduces the mean open time of these channels, and enrichment of the cells with CHS-containing vesicles attenuates this reduction (Lechleiter et al., 1986). Given the similarity of EtOH and halothane, it is feasible that CHS may exert a similar effect on the BKCa channel response to EtOH.

General Experimental Approach

The reductionist planar lipid bilayer system is well suited to address these questions. This system, described in detail in Chapter I, allows experimental control over both the membrane lipid environment and the channel reconstituted into it. The human brain clone of the BKCa channel α-subunit (hslo) is implemented in these studies, limiting complications possible with native channels such as differences in splice variants. This variant is most relevant to the human physiology of alcohol use. HEK293 cells serve as
the expression system, ideal since they lack any endogenous Ca\(^{++}\) sensitive conductances (Yu and Kerchner, 1998; Zhu et al., 1998). Channels are reconstituted into bilayers consisting of phosphatidylethanolamine (PE) and phosphatidylserine (PS) mixed in a 3:1 ratio, a mixture widely used in BK\(_{Ca}\) reconstitution experiments (Chu et al., 1998; Moczydlowski and Latorre, 1983a). This mixture will represent a baseline for basal channel function and EtOH sensitivity, to which the influence of added lipids (i.e. CHS) can be tested on these parameters.

Organization of the Thesis

Chapter I provides a detailed description of the planar bilayer method, as it is applied to the study of the actions of EtOH on ion channels. Chapter II details the CHS modulation of both basal \(P_o\) and the EtOH sensitivity of the BK\(_{Ca}\) channel cloned from human brain (\(hslo\)). Chapter III explores the mechanism underlying CHS modulation of channel function, in particular the role of surface charge, lipid molecular shape, and bilayer order. The discussion sections contained within each chapter summarize the findings and any mechanistic insight gained. A final Discussion chapter briefly summarizes the results and more general implications of the thesis work. Appendix I includes preliminary data addressing the role of [Ca\(^{++}\)]\(_{\text{free}}\) in the response of reconstituted \(hslo\) channels to EtOH.
CHAPTER I

ARTIFICIAL BILAYER TECHNIQUES FOR ION CHANNEL STUDY

Introduction

Many of the early studies of alcohol’s effects in the nervous system have been framed and interpreted to determine whether the primary target of alcohol’s action is the lipid or the protein components of brain. We suggest that it is necessary to consider functioning membrane proteins and their lipid environment (as well as the various interfaces between them, such as lipid-protein, lipid-protein-water, and protein-lipid-protein, etc.) as a dynamic system, in which the small amphiphilic alcohol molecule will interact simultaneously with a number of targets.

That lipids play an important role in the effects of alcohol on neural function is suggested by the great diversity of lipids in nerve membranes, the large influence of lipid composition on channel protein function, and by the fact that apparent compensatory changes in lipid composition occur as a function of chronic drug exposure. There is now a vast body of work that addresses the influence of chronic ethanol exposure on membrane lipid composition and function. However, results from different laboratories are often at odds. The complexity of natural membranes and the numerous and interlinked lipid metabolism pathways make a reasonable analysis of lipid involvement in alcohol’s actions in intact animals and tissue difficult.

Understanding how the interactions between protein subunits, lipids, and water are affected by alcohol is best accomplished in a very simplified system, where the function of an isolated channel protein is studied in a reconstituted planar lipid bilayer.
This technique has proven to be a very powerful method to elucidate the role of lipids in channel modulation. If we couple the use of the artificial membrane with the incorporation of cloned channels, we are able to manipulate both the protein and the lipid environment, yielding the greatest hopes of controlling enough variables to allow a meaningful assessment of the role of lipid environment on drug action on protein targets.

Building A Planar Lipid Bilayer Setup

Due to the fragile nature of the lipid bilayer, and the need to maintain an adequate signal-to-noise ratio, the recording chamber must be free of electrical interference, vibrations, and mechanical disturbances. As such, it is important to construct the bilayer rig in an area of the laboratory with little foot traffic and background noise. To eliminate floor vibrations, place the recording chamber on a vibration isolation table. The table is constructed of a large metal top that is supported on a bed of compressed nitrogen gas. They are commercially available from several sources, including Technical Manufacturing Company (Peabody, MA) and Kinetic Systems (Boston, MA). Cost-effective alternatives include placing the metal slab on a partially inflated motorcycle inner-tube (Alvarez, 1986), tennis balls, or pneumatic shock absorbers (Hanke and Schlue, 1993b). To eliminate electrical interference the chamber is placed in a Faraday cage, along with the probe of the patch-clamp amplifier used to measure the currents. The cages are also commercially available, but adequate homemade versions can be constructed. Cages can be built inexpensively with wood frames and copper or aluminum mesh, or from plate aluminum attached directly to the vibration isolation table. Additionally, the recording chamber is placed in an aluminum box with a closing top to
further reduce electrical interference. Large cages are convenient when a microscope is required for viewing the chamber. It is recommended that any piece of equipment that will contact the chamber, notably the perfusion system, also be shielded within the Faraday cage. Finally, the interior of the Faraday cage can be lined with sound-deadening foam, to prevent the bilayer recording setup from picking up acoustic noise from laboratory equipment and personnel.

Bilayer recording chambers come in a variety of styles. In this section, only chambers for use with painted bilayers will be considered. Figure 4 shows schematic drawings of two types of bilayer recording chambers. The chambers are commonly milled from a block of Teflon or Delrin plastic. Vertical bilayer chambers consist of two intersecting circular holes, one of which is fitted with a polycarbonate cup. The cup contains the aperture across which the bilayer is painted. The other chamber is constructed with a clear window to allow visualization of the bilayer with a microscope. Horizontal bilayer chambers contain two holes milled on either side of the plastic block, separated by a thin layer of plastic with a hole in the center. The bottom chamber is enclosed with a glass microscope slide, affixed to the Teflon/Delrin using a silicone sealant. A plastic microscope coverslip is attached to the bottom of the upper chamber. This coverslip acts as the divider between the two chambers and contains the aperture that will hold the bilayer. A 7:3 mixture of wax:Vaseline is used to attach the coverslip to the plastic chamber. Both chamber styles feature wells to insert the Ag/AgCl electrodes, and salt bridges that connect them to the recording chambers. In addition, ports can be added to attach perfusion and vacuum lines. Perfusion of the bilayer should
Figure 4. Schematic of Bilayer Recording Chambers
be designed in a way that minimizes the mechanical disturbance of the chamber. When working with horizontal bilayer chambers, a gravity-driven setup combined with a vacuum line is sufficient. The perfusion input and the vacuum line are placed on opposite sides of the chamber, and the rate of perfusion is best maintained below 1 ml/min. It is easier to perfuse the grounded chamber, whether upper or lower, as even a shielded perfusion line can act as an antenna. This method of bilayer perfusion produces noise in the current trace, and is useful mainly for bath exchange of the chamber during breaks in the recording period. If low-noise records are required during the perfusion of the chamber, a more elaborate perfusion system is required. Hanke and Schlue present a push/pull motor driven syringe system that is designed to maintain the volume of the chamber very precisely during perfusion (Hanke and Schlue, 1993b).

The apertures in the polycarbonate cups or plastic coverslips can be formed using a technique developed by Wonderlin, Finkel and French (Wonderlin et al., 1990). A small metal cone, or stylus, is attached to a power supply, used to heat it. A foot pedal is used to activate the heating element, leaving both hands free for manipulating the plastic. The cup or coverslip is pressed against the heated metal cone until a small depression forms. Once the depression is formed, the foot pedal is released and the plastic is held in place manually until it cools. After cooling, the cone-shaped depression in the cup/coverslip is shaved with a razorblade, under a microscope, to produce a circular hole. It is important to hold the blade level while shaving across the top of the cone. Use only apertures that are round, level, and smooth, to promote bilayer stability. Hole size is controlled by shaving at higher points on the cone, and working downward to increase
the diameter. Larger apertures will produce bilayers of larger capacitance, promoting incorporation, but yielding noisier recordings. Smaller holes allow more stable recordings, but the decrease in bilayer surface area reduces the incorporation efficiency. Holes on the order of 100 μm in diameter produce bilayers that yield a nice balance of stability and incorporation efficiency.

The electrodes in the chamber are attached to the headstage of a patch-clamp amplifier. Bilayer-specific models are commercially available, though any patch-clamp amplifier can be used, provided that the feedback resistance is on the order of 10 GΩ. The large capacitance of the bilayer produces considerable background noise, and allows only poor temporal resolution of voltage steps due to large capacitive transients. Bilayer-specific amplifiers circumvent this problem by implementing either integrating headstages or switching-resistive headstages that allow rapid charging of the bilayer membrane. A typical resistive-headstage patch amplifier will perform adequately when recording steady state activity of a channel at a particular voltage. A review of methods for maximizing bandwidth and the resolution of voltage steps, while reducing background noise, can be found in Chapter 5 of the Axon Guide (Sherman-Gold, 1993).

The design and analysis of electrophysiological experiments are commonly performed using a suite of software programs such as pClamp, designed by Axon Instruments. The software communicates directly with the amplifier, allowing manipulation of the holding potential, and the generation of voltage pulses. As a result, a DMA interface is required to allow crosstalk between the digital signals of the PC and the analog signals of the amplifier. The interface connects to the PC, and usually runs off of
the PC power supply. If extremely low-noise recordings are necessary, it may be beneficial to use an interface with an isolated power supply. The output of the amplifier can be stored directly on the computer, or on a tape recorder (Bezanilla, 1985). When the voltage protocol involves voltage pulses with short durations, storage directly onto the PC is acceptable. However, a tape recorder is recommended when obtaining long records of steady state channel activity at a particular voltage. Data can be stored directly onto a digital audio tape (DAT) recorder, or onto a Betamax tape with the use of a pulse code modulator (PCM) to convert the amplifier output to a digital signal. A DAT recorder designed to collect data in this fashion is available from Dagan Corporation (Minneapolis, MN). The advantages of this mode of recording include large storage capacity and ease of data retrieval, as records are stored as separate tracks on the tape. The Betamax/PCM combination works adequately, though Betamax tape availability is somewhat limited. An 8-pole low-pass Bessel filter is useful for resolving the data during an experiment, though it is best to store the data at the bandwidth that it leaves the amplifier. This allows the data to be filtered at a desired frequency when the records are played back from the tape and stored onto the PC. It is useful to view the voltage pulses and corresponding currents on an oscilloscope, both during the experiment and during playback of data stored on tape. Figure 5 is a general schematic of the electronics and connections of a typical bilayer recording setup.

Biochemical Preparations for Reconstitution

The planar bilayer technique has a number of advantages. It allows control of both the lipid environment of the channel, and the aqueous phases at the intra- and
Figure 5. Planar Lipid Bilayer Recording Setup
extracellular face of the channel. In addition, reconstitution of channel proteins from biochemical preparations circumvents the issue of accessibility when studying channels in membranes not easily patched with a glass micropipette. Investigators can isolate a native channel from a specific tissue, or transfect a cell line with a clone of interest. The use of cloned channels adds another level of control, since manipulation of the channel protein is possible before insertion into the bilayer. Crude membrane fractions containing the channel of interest are suitable for incorporation into artificial membranes. Below, a procedure is outlined for isolating both native and cloned channels. The channel type discussed in each case is the large conductance Ca\(^{++}\)-activated K\(^+\) channel (BK\(_{Ca}\)), studied extensively using reconstitution techniques. This channel represents, for the ethanol researcher, a functionally relevant and easily accessible target. Data from our laboratory have demonstrated both acute (Dopico et al., 1996; Dopico et al., 1998; Dopico et al., 1999) and chronic (Knott et al., 2002) channel regulation by ethanol. In addition, acute regulation of the drug persists in the planar lipid bilayer, when addressed using both native (Chu et al., 1998; Walters et al., 2000) and cloned (Crowley et al., 2000) channels. The crude membrane fractions derived from these sources yield a plentiful supply of channel proteins that readily incorporate into painted bilayers. The reader is encouraged to refer to the original citations for experimental detail, as well as a recent Methods in Enzymology chapter that covers ion channel reconstitution (Favre et al., 1999).

*Rat Skeletal Muscle T-tubule Preparation*
A protocol for isolating T-tubule membranes from rabbit skeletal muscle was first described Rosemblatt and colleagues (Rosemblatt et al., 1981). This technique was adapted by Moczydlowski and Latorre to isolate rat skeletal muscle T-tubules (Moczydlowski and Latorre, 1983b), from which they described in detail the gating kinetics of a Ca\textsuperscript{++}-activated K\textsuperscript{+} channel reconstituted into planar lipid bilayers (Moczydlowski and Latorre, 1983a). This preparation was also used to quantitate differences in BK\textsubscript{Ca} channel activity in bilayers composed of neutral phosphatidylethanolamine (PE) versus negatively-charged phosphatidylinerine (PS) (Moczydlowski et al., 1985). This study demonstrates not only the importance of bilayer surface charge for channel function, but that lipid exchange occurs between the artificial membrane and the incorporated membrane fragments. In our laboratory, rat t-tubule BK\textsubscript{Ca} channels incorporated into PE/PS (3:1 w/w) bilayers show a dose-dependent increase in activity upon addition of ethanol (Chu et al., 1998). The reader is encouraged to obtain detailed methodology from the references above.

**Crude Membrane Fractions from Cultured Cells**

The expression of cloned channels in cultured cells provides a number of experimental advantages for reconstitution experiments. The high protein expression levels and relative ease of culture maintenance provide an abundant supply of channels for incorporation. Experiments undertaken with cloned proteins also have the obvious advantage of manipulation of the protein sequence before expression and reconstitution.

HEK-293 cells are a common line used for expression of cloned BK\textsubscript{Ca} channels. The cells express an array of endogenous chloride and potassium channels, but no Ca\textsuperscript{++}-
dependent currents are detected. In addition, neither BKCa blocker charybdotoxin nor small-conductance Ca\(^{2+}\)-activated K\(^+\) channel (SK) blocker apamin can inhibit endogenous outward currents (Yu and Kerchner, 1998; Zhu et al., 1998). Therefore, confirmation that a cloned channel has incorporated into a bilayer is easily accomplished by altering the free Ca\(^{2+}\) in the recording solution. Chinese hamster ovary (CHO) cells and COS cells are also commonly used as expression systems.

The array of endogenous channels, ease of transfection, and the protein expression levels are all important considerations when choosing a cell line to express a cloned channel for reconstitution. An effective protocol for isolation of membrane fragments from cultured cells is described by Sun, Naini, and Miller (Sun et al., 1994). A variation of this protocol, implemented during the thesis work, is described in the Materials and Methods section of Chapter II, and the accompanying Figure 10.

*Lipids used in Reconstitution Experiments*

The lipids used for bilayer reconstitution experiments are commercially available from Avanti Polar Lipids (Alabaster, AL), in both natural and synthetic form. The lipids are purchased either as a powder or dissolved in chloroform. For a reconstitution experiment, an appropriate phospholipid mixture is aliquoted and mixed, under N\(_2\) gas. The selection of the lipid mixture is influenced both by the experimental design and by the need to promote vesicle incorporation. The lipid mixture is vortexed and dried under N\(_2\) gas. The dried lipid is resuspended at the desired concentration in decane. The decane solvent is air-sensitive and should be stored under N\(_2\) gas.
Most reconstitution experiments employ mixtures of phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylcholine (PC). PE/PS bilayers mixed in a 3:1 (w/w) ratio are stable, and readily incorporate channels from both membrane preparations described above. In general, mixtures should contain PE and acidic lipids such as PS and phosphatidylinositol (PI) to promote vesicle incorporation. In addition, certain channels require specific lipid species to function properly, such as the requirement of cholesterol for the nicotinic acetylcholine receptor (Barrantes, 1989). These are important factors to consider when choosing the bilayer composition.

Experimentally, diverse lipids are often used as probes to investigate their influence on ion channel function through alterations in physical properties such as the headgroup size and charge, as well as the degree of acyl chain saturation. Figure 6 displays the names and structures of the lipids implemented in the experiments of this thesis.

Recording from the Planar Lipid Bilayer

To prepare the cup/coverslip for a reconstitution experiment, the aperture is pretreated with the lipid mixture dissolved in decane. A sable hair paintbrush, size 000 or 0000, is used to paint a drop of the mixture across the hole. The brush is most effective when only two bristles, positioned directly next to one another, are left in the tip. To prepare the brush, use a microscope and a pair of microdissection scissors to snip the bristles. To reduce contamination across experiments, a separate brush should be prepared for use with a particular lipid mixture. Capillary action allows a small amount of the decane/lipid mixture to stick to the bristles, which is deposited across the aperture. The drop should fill the entire opening, without flooding the coverslip. Over the course
Figure 6. Structures of the Lipids Employed in the Experiments of this Thesis

1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE)

1-palmitoyl-2-oleoyl-phosphatidylserine (POPS)

1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC)
Figure 6 (cont’d.). Structures of the Lipids Employed in the Experiments of this Thesis
of one minute, the drop will dry and leave a rim of dried lipid around the edge of the hole. Allow the cup/cover slip to dry for 5 minutes before recording.

Once the coverslip is dry, the recording chamber can be assembled with the electrodes and salt bridges. Both chambers are filled with the appropriate recording solutions, and the amplifier is switched on. The lipid mixture is again brushed across the aperture. Instead of a paintbrush, a glass pipette with a rounded tip (formed by rotating the pipette over a Bunsen burner) is used. Once cooled, the bulb is dipped in the decane/lipid mixture, and brushed across the aperture.

While attempting to form a bilayer, a repetitive triangular voltage waveform is maintained across the aperture to monitor bilayer formation. The amplitude and rate is set to a desired value (20 mV/25 ms, for example). The capacitance of the bilayer can be determined using the equation \( I = (C)\frac{dV}{dt} \), where \( I \) is the capacitive current amplitude, \( C \) is the capacitance, and \( \frac{dV}{dt} \) corresponds to the change in voltage over time (known from the size and duration of the triangle pulse). Before recording from the bilayer, the triangle pulse is presented using a series of known capacitors. The size of the resulting capacitive current is plotted against the known capacitance to produce a standard curve. This is used to determine bilayer capacitance when the triangle pulse is run across the artificial membrane. The size of the current will be proportional to the size of the aperture, and it should be very square in shape. The capacitive current should retain its size and shape for several minutes before adding the channel preparation.

The method for incorporation of channel proteins is slightly different in vertical versus horizontal bilayer chambers. For horizontal chambers, a pipette is used to drop
0.5 µl of channel preparation above the aperture. A long gel-loading pipette tip is recommended, as it allows a slow, controlled release of the membrane preparation. The force of gravity will pull the channel-containing fragments down onto the bilayer. For vertical bilayer setups, a stirring mechanism is required to drive channel incorporation. Regardless of the chamber type used, there are a number of experimental conditions that will promote channel incorporation. The lipid mixture used to form the bilayer should contain PE, and some proportion of negatively charged lipids such as PI or PS. The cis chamber, to which the membrane preparation is added, should contain some free Ca$$^{++}$$, and be hyperosmotic relative to the trans chamber (Miller et al., 1976; Miller and Racker, 1976). In cases where vesicles or liposomes are added, they should be prepared such that the interior is hyperosmotic relative to the bath solution. The mechanisms underlying these requirements are not completely understood, and remain rigorously studied since vesicle fusion plays a vital role in many biological processes. All of these conditions can be optimized for a given membrane preparation, with some trial and error. More extensive explanations of the principles of channel incorporation are available in reviews by Labarca and Latorre (Labarca and Latorre, 1992), and chapters in Ion Channel Reconstitution (Hanke, 1986), and Planar Lipid Bilayers: Methods and Applications (Hanke and Schlue, 1993a).

Once channel openings appear in the bilayer recordings, further incorporation events can be prevented by neutralizing the osmotic gradient and/or dropping the [Ca$$^{++}$$]$$_{\text{free}}$$ in the cis chamber. This can be accomplished by perfusion, or by addition of salts and/or Ca$$^{++}$$-chelators to the appropriate chamber. In cases where multiple channel
openings are visible, the number of channels can be determined by manipulating conditions (such as membrane potential) to maximize the open probability ($P_o$) of the channel. At positive potentials, the likelihood of all channels simultaneously entering the open state is very high, and the number of channels can be determined by computing the number of elemental contributions necessary to reach the cumulative current. It is useful to record the steady state channel activity at a number of voltage steps before testing the effects of ethanol to ensure that basic channel properties, such as conductance and voltage sensitivity, are normal. The stability of the bilayer is dependent upon the lipid mixture, recording conditions, and the size and shape of the coverslip onto which the bilayer is painted. These parameters can all be optimized through trial and error to yield stable recordings lasting minutes, sufficient to produce a wealth of single channel data for analysis.

Before adding ethanol, a control record should be recorded for at least one minute at a particular voltage. It is important that the activity of the channel is stable, as any rundown or increase can skew the effects of the drug. After the addition of ethanol, periodically monitor the capacitive current to determine the stability of the bilayer. Record the channel activity at several voltage steps in the presence of ethanol, to allow comparison of the slope conductance and voltage sensitivity in the presence and absence of the drug.

**Analysis of Single Channel Data**

Data stored on a tape recorder is re-acquired in the gap-free recording mode of a program such as Fetchex, available in the pClamp suite of software from Axon.
Instruments. It is recommended that data generated during reconstitution experiments be stored unfiltered, at the bandwith that it leaves the amplifier (10 kHz, for example). The records can be passed through an 8-pole low-pass Bessel filter during re-acquisition, and filtered at a desired frequency. This allows one record to be used for detailed kinetic analysis, by resolving short events with minimal filtering, or to be low-pass filtered at a lower frequency for display.

Records of steady-state single channel activity yield a wealth of information. Programs such as Fetchan, another pClamp module, are designed to facilitate the analysis of the data. The first step in analyzing a channel record is the construction of an all-points amplitude histogram. The histogram will show a peak, with a Gaussian distribution, for both the closed and open state of the channel. A least-squares function is used to fit the histogram generated from the channel activity record at a given voltage. This will yield both the open probability ($P_o$) of the channel, as well as the amplitude of a single channel event ($i$) at that voltage. This exercise is repeated at a number of holding potentials, so that both $i$ and $P_o$ can be plotted against voltage. From this, both single channel conductance ($g$) and voltage sensitivity can be determined.

Comparison of the channel $P_o$ in the presence and absence of ethanol, under otherwise identical conditions, provides a straightforward means of assessing the overall effect of the drug on channel activity. In single-channel bilayers, the analysis can be carried a step further by constructing and analyzing an events list. An events list is displayed as a histogram with the number of observations plotted versus event duration. This histogram is fit with a series of exponential functions, using a maximum-likelihood
estimator, to uncover the time constants for a particular channel state. An F-statistic table is used to determine the minimum number of exponentials that adequately fit the histogram. In practical terms, an open state histogram that is best fit with two exponentials indicates there are a minimum of two kinetically distinct channel openings, a short and a long open state. The fit of the histogram provides not only the duration of these kinetic states, but also the proportion of time the channel spends in them. The analysis is performed for both the open and closed events. Events lists can be generated from channel records before and after the addition of ethanol. Comparison of this data demonstrates how the drug modifies the open and closed kinetic states of the channel.

While studying ethanol modulation of single channels can yield a wealth of information, it may also produce significant variability in a data set. It is important to bear in mind that whole cell electrophysiology techniques average the behavior of many ion channels during a single sweep in any given experiment. This minimizes differences in the EtOH response within a channel population that may result from, for instance, differences in post-translational modifications or other unknown factors. For this reason, most figures in this thesis addressing the EtOH response of single channels in a given bilayer type are scatter plots. These plots show the response of each experiment in the data set to convey the variability in the single channel responses.

Examples of Data Obtained Using the Planar Lipid Bilayer

Figure 7 and Figure 8 contain representative traces of reconstituted hslo BK<sub>Ca</sub> channels in POPE/POPS (3:1) bilayers, demonstrating that characteristic voltage sensitivity (Figure 7), large conductance (Figure 7), and Ca<sup>++</sup> sensitivity (Figure 8) are
Figure 7. Reconstituted BK$_{ca}$ channels exhibit characteristic large conductance and retain sensitivity to membrane depolarization in the planar lipid bilayer. Records were obtained in POPE/POPS (3:1) bilayers at the indicated holding potentials, with 50 μM [Ca$^{2+}$]$_{ic}$ in the bath. The bilayer contains 1 channel, and the arrows denote the closed state. Data was low-pass filtered at 1 kHz and digitized at 10 kHz. A current – voltage plot, well fit by a linear function, indicates the large-conductance of the channel.
Figure 8. Reconstituted BKCa channels exhibit characteristic Ca\(^{++}\) sensitivity in the planar lipid bilayer. A) Records were obtained in separate POPE/POPS (3:1) bilayers at 0 mV, with the indicated [Ca\(^{2+}\)]\(_{ic}\) in the bath. The bilayers contain 1 channel, and the arrows denote the closed state. Data was low-pass filtered at 1 kHz and digitized at 10 kHz. B) Plot summarizing the open probability versus [Ca\(^{2+}\)]\(_{ic}\) data.
Figure 9. Representative traces of cloned hSlo BK<sub>Ca</sub> channels incorporated into a POPE/POPS (3:1) bilayer in the absence and presence of 50 mM EtOH. Records were obtained at 0 mV, with ~25 μM [Ca<sup>2+</sup>]<sub>e</sub> in the bath. The bilayer contains 2 channels, and the arrows denote the closed state. Data was low-pass filtered at 1 kHz and digitized at 10 kHz.
retained following reconstitution of the channel. Figure 9 shows representative traces of a cloned *hslo* BKca channel before and after the addition of 50 mM EtOH. Ethanol enhances the activity of the cloned channel to a similar extent to that seen for the native rat t-tubule channel. These and previous (Chu *et al.*, 1998) data demonstrate that the BKca protein (or protein complex), in the absence of complex lipid architecture or cytoplasmic elements, is capable of responding to ethanol in a manner similar to that of the channel *in situ*, and makes obvious the ability to manipulate the lipid environment and assess the consequences on drug action.

**Advanced Applications - Asymmetric bilayers**

Most of the planar bilayer work described in the literature, and above, utilizes symmetric bilayers, in which each of the leaflets is identical. However, biological membranes are not typically symmetrical, and a truer representation of drug action on membranes and associated proteins will involve the generation of asymmetric bilayers. Described simplistically, the formation of asymmetric bilayers is often accomplished by the apposition of two monolayers in troughs separated by a movable partition. As the partition is removed, the monolayers are forced together to form an asymmetric bilayer, often referred to as a folded bilayer (Cassia-Moura *et al.*, 2000; Heywang *et al.*, 1998; Montal and Mueller, 1972; Tancrede *et al.*, 1983). It is important to recognize that the final organization of the folded bilayer may not represent total segregation of the monolayer components from each other.

**Supported bilayers**
Another “advanced” technique involves the formation of the bilayer on solid or semi-solid platforms, enhancing their stability and allowing conditions and measurements not possible with the previously described painted bilayer. These include ordering the lipids in the bilayer (Heywang *et al.*, 1998), simultaneous electrical and optical measurement of single ion channels (Ide and Yanagida, 1999), and the use of a polymer support platform associated with tethered bilayer-imbedded proteins, allowing lateral movement and monitoring of the protein (Wagner and Tamm, 2000).

**Conclusions**

The techniques and data described in this chapter illustrate artificial planar bilayer techniques, and demonstrate how these techniques can be used to study the influence of the lipid environment on protein function and drug action. We have focused on electrophysiological measurements of neuronal channels incorporated into bilayers. The techniques of planar bilayer formation and protein incorporation must be practiced before they become routine, and contain enough unknowns to still be something of an art. However, once incorporation and recordings are obtained, the analysis of the emerging records is not different from those of any single channel experiment utilizing patch clamp technology. Of course, the nature of the artificial bilayer experiment is very reductionist, and as with any reductionist experiment, caution must be exercised in the interpretation of results, since proteins (probably) do not exist in one or two-lipid environments. However, the information obtained from these simple experiments can be used to derive hypotheses testable in more complicated lipid environments.
Reference List


CHAPTER II

CHOLESTEROL ANTAGONIZES ETOH POTENTIATION OF HUMAN BRAIN

BK_{Ca} CHANNELS IN PHOSPHOLIPID BILAYERS

Introduction

Ion channels reside in a heterogeneous lipid matrix. Lipid species partition asymmetrically both within and across biological membrane leaflets (Devaux, 1991). These non-random lipid associations produce domains that differ in composition and physico-chemical properties from the bulk membrane (Welti and Glaser, 1994), resulting in distinct microenvironments for ion channels. An example is the cholesterol (CHS) and sphingomyelin-rich lipid raft, thought to participate in many aspects of cell function (Brown and London, 1998). Large conductance, Ca^{++}-activated K^{+} (BK_{Ca}) channels cloned from human brain (hslo) and expressed in MDCK cells associate with these lipid microdomains (Bravo-Zehnder et al., 2000). The biological implications of channel association with distinct lipid domains are not well understood, but it is likely that the domain physical properties influence channel activity.

Cholesterol is a significant component of lipid rafts and a major determinant of overall membrane physical properties (Bloch, 1983), which may contribute to its effects on the activity of native ion channels (Bo alotina et al., 1989; Barrantes, 1993; Chang et al., 1995b; Lundbaek et al., 1996; Levitan et al., 2000). Modulation of membrane CHS content and distribution may play a role in cellular adaptation to ethanol (EtOH). Increased content (Chin et al., 1978; Omodeo-Sale et al., 1995) and altered distribution
of CHS between membrane leaflets (Wood et al., 1990a) are observed in response to EtOH exposure in both animal and cell culture models. Ethanol differentially increases the diffusion of various lipid probes in Aplysia neurons, suggesting that its actions on ion channels might depend upon the existence of dissimilar lateral domains (Treistman et al., 1987). In model membranes and computer simulations, EtOH affects lateral lipid domains (Chin and Goldstein, 1981; Harris et al., 1984b; Jorgensen et al., 1993; Slater et al., 1993) suggesting that specific lipid species might modify the sensitivity of a domain to EtOH action. Cholesterol, in particular, counteracts EtOH’s disordered action in mouse synaptosomal membranes and phospholipid bilayers (Chin and Goldstein, 1981). Interestingly, EtOH increases the fluidity of the extracellular leaflet of synaptic plasma membranes to a larger extent than that of the cytoplasmic leaflet, an effect attributed to the larger CHS content in the inner leaflet. After chronic EtOH treatment, however, transbilayer differences in fluidity and CHS content are reduced in concert (Wood et al., 1990a).

Ethanol reversibly potentiates BKCa channel activity in excised membrane patches from rat neurohypophysial terminals, an action that may contribute to EtOH inhibition of neuropeptide release (Dopico et al., 1996). EtOH potentiation persists after expression of BKCa (mslo) channels in Xenopus oocytes (Dopico et al., 1998), and incorporation of native BKCa channels into 1-palmitoyl-2-oleoyl phosphatidylethanolamine (POPE)/1-palmitoyl-2-oleoyl phosphatidylserine (POPS) bilayers (Chu et al., 1998). Thus, neither cytosolic second messengers, nor complex cytoskeletal architecture are required for EtOH action on BKCa channels.
Here, we use *hslo* channels expressed in HEK 293 cells, and incorporated into bilayers made of only one or two phospholipid species to study CHS modulation of EtOH action on channel function. This preparation allows near complete control of both protein and lipid constituents. Results indicate that increased bilayer CHS antagonizes EtOH potentiation of channel activity in a concentration-dependent manner. The reciprocal actions of EtOH and CHS on commonly targeted channel dwell states are the major determinants of CHS antagonism of EtOH effect on channel activity. Furthermore, the effect of each of these modulators on channel activity is drastically reduced in the absence of phosphatidylserine in the bilayer.

**Materials and Methods**

**HEK 293 membrane preparation.** HEK-293 membrane fragments were isolated using a protocol for COS cells (Sun *et al.*, 1994), modified slightly. Briefly, HEK 293 cells stably transfected with *hslo* cDNA (a gift from Dr. P. Ahring, NeuroSearch A/S, Denmark) were grown to confluence, pelleted, and resuspended on ice in 10 ml of buffer (mM): 30 KCl, 2 MgCl₂, 10 N-2-hydroxyethylpiperazine-N' -2-ethanesulfonic acid (HEPES), 5 ethylene glycol-bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid (EGTA); pH 7.2. The cell suspension was forced through a 27-gauge needle 4 times, and sonicated at 30% maximum power for 30 sec, twice. The suspension was layered on a 20-38% sucrose density gradient (in 20 mM MOPS, pH 7.1) and centrifuged at 25,000 rpm for 60 min at 4° C. The band at the 20%-38% interface was collected with a syringe, diluted with bidistilled H₂O, and centrifuged in a 50.2 Ti rotor at 45,000 rpm for 60 min at 4° C. The resulting pellet was resuspended in 200 µl of buffer (mM): 250 sucrose, 10 HEPES;
pH 7.3. Aliquots were stored at -80°C. Figure 10 is a schematic representation of the isolation and reconstitution of the HEK-293 membrane fragments.

Electrophysiology. Channels were incorporated by dropping 0.5 μl of the membrane preparation onto bilayers consisting of POPE/POPS and differing concentrations of CHS. Lipids were dried under N₂ gas, and resuspended in decane in a 3:1 (w/w) POPE/POPS ratio, with 0–49 mol% CHS. The final lipid concentration was 25 mg/ml. Bilayers were formed by painting the lipid mixture across a 100 μM hole formed in a plastic coverslip (Wonderlin et al., 1990). Capacitance was monitored by the capacitive current generated by 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 their Ca²⁺-sensor facing the cis chamber were studied. Solutions consisted of (mM): cis, 300 KCl, 10 HEPES, 1.10 N-(2-hydroxyethyl) ethylene-diaminetriacetic acid (HEDTA) ([Ca²⁺]ₜₐₐₜ 50 μM) or 1.45 HEDTA ([Ca²⁺]ₜₐₐₜ 15 μM), 1.05 CaCl₂, pH 7.2, and trans, 30 KCl, 10 HEPES, 0.1 HEDTA, pH 7.2. CHS action on baseline channel function was studied at 50 μM [Ca²⁺]ₜₐₐₜ, and 0 mV, which promotes high channel activity, from which the inhibitory effects of CHS can be effectively quantitated. EtOH sensitivity was tested at 10 μM [Ca²⁺]ₜₐₐₜ, at potentials between -10 mV and +60 mV, which yields activity low enough to prevent a “ceiling effect” (reaching maximal channel NPO) when measuring EtOH potentiation. The magnitude of EtOH activation of slo activity (NPO, EtOH/NPO Control) is independent of voltage within this range (Dopico et al., 1998). [Ca²⁺]ₜₐₐₜ in the cis chamber was adjusted using aliquots from a 1 M stock solution of HEDTA (pH 7.2) [Ca²⁺]ₜₐₐₜ values given are nominal, calculated
Figure 10. Isolation and reconstitution of HEK-293 membrane fragments.
with the Max Chelator Sliders program (C. Patton, Stanford University).

Experiments were performed at room temperature (20-25 °C) to facilitate comparison with our previous studies examining EtOH action on BKCa channels (Dopico et al., 1996; Dopico et al., 1998; Chu et al., 1998). 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 either 3 (for dwell time analysis) or 1 kHz (for display and NP determination) 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 6.0.2 (Axon Instr., 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 (Po). N was monitored pre- and post-EtOH by stepping to positive potentials to maximize Po. Experiments showing an increase in N after EtOH addition were discarded. NPo was determined from periods of at least 20 sec of continuous recording. Dwell time histograms were constructed using the half-amplitude threshold criterion, events shorter than 0.3 ms being excluded. An F table (p<0.01) was used to determine the minimum number of exponential components to appropriately fit dwell-time histogram data. Fifty percent of maximal effect (EC50 or IC50) was obtained from concentration-response curves by extrapolation. Data are shown as mean±S.E.M. The significance of the difference between means was determined by ANOVA and test a posteriori (Dunnett’s).
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, and CHS from Avanti Polar Lipids (Alabaster, AL).

Results

Ethanol increases hslo channel activity in binary bilayers. We first determined whether EtOH modifies hslo steady-state activity (NP₀) in a binary phospholipid mixture. We chose a 3:1 POPE/POPS (w/w) planar bilayer, where EtOH sensitivity of native skeletal muscle BKCa channels was initially explored (Chu et al., 1998). Thus, cloned hslo subunits were incorporated into this bilayer type, where they displayed characteristic features of BKCa channels: large unitary slope conductance (∼330 pS, Figure 7), and increases in P₀ as the applied voltage is made more positive (Figure 7) (9.8 mV±0.4 mV/e-fold change in NP₀ (n= 12)) and/or [Ca++] at the cytosolic side of the channel is increased (Figure 8). Figure 11 shows traces of hslo activity before and during application of 50 mM EtOH to the “intracellular” side of the POPE/POPS bilayer. The EtOH-induced increase in P₀ shown in the figure, which occurred within 1-5 min of drug exposure, was observed in 8 out of 10 bilayers, the average NP₀ showing a 5.2±1.5 fold increase over pre-EtOH values. EtOH-induced potentiation of hslo channel activity in the POPE/POPS bilayer is similar to our previously reported results in complex lipid/protein systems with mslo subunits expressed in oocytes (Dopico et al., 1998), native BKCa channels studied in situ (Dopico et al., 1996), and rat skeletal muscle t-tubule BKCa channels reconstituted into this bilayer type (Chu et al., 1998).
Figure 11. Ethanol (50 mM) increases hslo channel activity in planar POPE/POPS bilayers. Traces of hslo activity recorded before (left) and during (right) application of EtOH to the intracellular (cis) side of the same POPE/POPS 3:1 (w/w) bilayer. The potential across the bilayer was set at 0 mV and free [Ca$^{++}$]$_{ic}$ 10 μM. Data were low pass filtered at 1 kHz and sampled at 10 kHz. Arrows denote the current level corresponding to channel closed states. Steady-state channel activity (NPo) was determined from continuous recording (see Materials and Methods).
Thus, \textit{hslo} subunits, POPE, POPS, and the system interfaces are sufficient for EtOH potentiation of BK_{Ca} channel activity.

In contrast to its action on steady-state activity, 50 mM EtOH consistently failed to modify other parameters of \textit{hslo} function, such as unitary amplitude (14.9 vs. 14.8 pA, recorded at 0 mV and 300/30 mM \text{[K']} /[\text{K'}]_o in the presence and absence of EtOH, respectively) (Figure 11), suggesting that EtOH actions on \textit{hslo} channels are restricted to modification of channel gating. This is also in agreement with data obtained with BK_{Ca} channels in more complex lipid/protein systems (Dopico \textit{et al.}, 1996, 1998; Chu \textit{et al.}, 1998; Jakab \textit{et al.}, 1997), validating our minimal system for studies on CHS modulation of EtOH sensitivity.

\textbf{Cholesterol blunts alcohol potentiation of \textit{hslo} channel activity.} We next tested whether CHS incorporation into this binary bilayer could modify EtOH-induced increases in \textit{hslo} activity. Figure 12 shows that CHS incorporation (11-33 mol\%) into POPE/POPS bilayers diminishes EtOH-induced potentiation in a concentration dependent manner (IC$_{50}$= 15 mol\%). CHS content of 23 mol\% resulted in almost total prevention of channel potentiation by 50 mM EtOH. Under these conditions, EtOH slightly increased channel activity in 2 out of 5 experiments, and slightly inhibited activity in the remaining 3 cases, yielding no net effect on channel activity (average NP$_o$ value=0.96±0.38 of controls). This lack of a major alcohol effect in the presence of CHS may be explained by: 1) reduced EtOH partitioning into CHS-containing bilayers, 2) CHS antagonism of EtOH at EtOH’s recognition site(s) on the \textit{hslo} subunit or its immediate phospholipid.
Figure 12. Cholesterol antagonizes EtOH-induced potentiation of hslo channel activity in POPE/POPS 3:1 (w/w) bilayers. The plot shows that average ratios of hslo NP₀ in the presence and absence of 50 mM EtOH are progressively decreased as the bilayer CHS content is increased. EtOH potentiation is significantly reduced at ~23 mol% [CHS] (IC₅₀=15 mol%). The potential at the cis side of the bilayer was set between -10 and +20 mV, and free [Ca²⁺]₀ = 10 µM. Data were low pass filtered at 1 kHz and sampled at 10 kHz. NP₀ values were determined from continuous recording (see Materials and Methods). Data are means±SEM; n= 2–8 bilayers. *Significantly different from POPE/POPS (control) values (p<0.05; ANOVA and Dunnett’s test) POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; POPS; 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine; CHS, cholesterol.

of basal channel activity.
microenvironment (see Discussion), and/or 3) the actions of CHS and EtOH on channel kinetic states are of opposite sign, and balanced, which results in reciprocal modulation

**Cholesterol reduces basal hslo channel activity in POPE/POPS bilayers.** A reduction in hslo activity caused by the presence of CHS in the bilayer could explain part or all of the CHS antagonism of EtOH potentiation of hslo activity in POPE/POPS bilayers.

Figure 13a shows hslo single channel activity recorded under identical conditions of voltage and free [Ca^{2+}]_c at the cis side of POPE/POPS bilayers in the absence and presence of CHS in this bilayer. Increases from 11 to 49 mol% inhibit hslo activity in a concentration-dependent manner. Maximal inhibition is reached at 33-49 mol% CHS with an IC_{50}=15.5 mol% (Figure 13b). Both the concentration for maximal effect and the IC_{50} are similar to those for CHS blunting of EtOH-induced increases in hslo activity (see above), which suggests that CHS inhibition of channel basal activity contributes to the sterol modulation of alcohol-induced potentiation of hslo activity.

Inhibition of basal P_o could result from a CHS-induced decrease in t_o, an increase in t_c, or a combination of both. Figure 13c and 13d clearly demonstrates that the third possibility is the case. Furthermore, single channel analysis reveals differential CHS effects on t_o and t_c, dependent upon concentration. While inhibition of channel t_o is maximal at 23 mol% CHS (IC_{50}=4.3mol%) (Figure 13c), channel t_c failed to reach a well-defined maximum at the concentrations tested (Figure 13d). If a maximal effect is assumed at the highest [CHS] tested (49 mol%), t_c data extrapolation yields an EC_{50}=36.1mol%, representing a minimum for this value. Thus, at concentrations of CHS
Figure 13. Inhibition of basal hslo channel activity, and its underlying changes in mean open and closed times, as a function of cholesterol content in POPE/POPS 3:1 (w/w) bilayers. A) Single current traces displaying 2 sec of continuous recording obtained in the absence (top) and presence (bottom) of 49 mol% CHS. The potential at the cis side of the bilayer was set at 0 mV, and free [Ca^{2+}]_i 50 μM. Data were low pass filtered at 1 kHz and sampled at 10 kHz. Arrows denote the current level corresponding to channel closed state(s). POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; POPS; 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine; CHS, cholesterol; B) Increases in CHS content (11-49 mol%) inhibit hslo activity (P_o) in a concentration-dependent manner. Maximal inhibition is reached at 33-49 mol% CHS (IC_{50}=15.5); C) Channel mean open time (t_o) is maximally inhibited at 23 mol% CHS (IC_{50}=4.3); D) Channel mean closed time (t_c) fails to reach a well-defined maximum, but progressively increases as CHS content in the bilayer increases. If a maximal effect is assumed to be reached at the maximal [CHS] tested (49 mol%), data extrapolation renders EC_{50} 36 mol%. Both t_o and t_c were obtained by weighting the different exponential components of respective dwell-times distributions (Fig. 17). For B)-D), data are means±SEM; n= 2 – 8 bilayers; *Significantly different from POPE/POPS (control) values (p<0.05); **Significantly different from POPE/POPS (control) values (p<0.01) (ANOVA and Dunnett’s test).
below the IC₅₀ for the reduction in Pₒ, decreases in channel activity are determined by a major reduction in tₒ and a minor increase in tₑ. In contrast, above IC₅₀, further reduction in Pₒ is primarily due to a progressive increase in tₑ.

As with EtOH (see above), CHS-induced modifications of hslo channel Pₒ were not accompanied by significant changes in conductance (pS): 323.3±5 in POPE/POPS (n=12), 329.2±4 in POPE/POPS+13% CHS (n=11), 335.3±7.3 in POPE/POPS+33% CHS (n=4); all measurements obtained in 300/30 mM [K⁺]/[K⁺]ₒ (r² > 0.95 for linear fits of i/V data). This result is in agreement with studies of CHS action on BKₖa channels in rabbit aorta (Bolotina et al., 1989). A subtle 7% change in slope conductance has been reported for rat brain BKₖa channels incorporated into POPE/POPS (55:45) bilayers containing 11% CHS (w/w) (Chang et al., 1995), an effect not apparent in our system. Those experiments were conducted in the presence of a 300/100 mM (cis/trans) KCl gradient, while ours were performed with a larger 300/30 mM KCl gradient. This lower trans chamber [K⁺] in our experiments likely created a driving force large enough to mask subtle changes in slope conductance caused by CHS. In summary, CHS-induced reduction of hslo channel Pₒ occurred without major changes in unitary conductance, as in more complex systems. As for EtOH, the channel pore-forming hslo subunits reconstituted into a minimal bilayer system are sufficient for CHS modulation of BKₖa channel activity.

**Role of phosphatidylserine for the actions of EtOH and CHS on hslo channels.** We next tested the role of POPS in the modulation of channel activity by EtOH by casting bilayers from 100% POPE. The amount of PS is altered in synaptic plasma membranes
after EtOH treatment (Sun and Sun, 1985). Removal of POPS may alter lateral domain formation in the bilayer resulting from POPE-POPS and POPS-POPS headgroup interactions. Headgroup interactions and lateral domains may influence EtOH action (Treistman et al., 1987; Jorgensen et al., 1993), as well as CHS miscibility in the membrane (see Discussion).

POPS carries a net negative charge at physiological pH, at which our experiments were performed. Negative surface charge promotes adsorption of cations to the membrane (McLaughlin et al., 1981), and reduces BKCa channel conductance and P0 (Moczydlowski et al., 1985; Turnheim et al., 1999; Park et al., 2003). As expected, hslo channels displayed changes in slope conductance consistent with the loss of negative charge carried by POPS: 323.3±5.8 (n= 12) vs. 281.8±11.1 pS (n= 14) (p<0.004), in POPE/POPS (3:1; w/w) vs. POPE bilayers, respectively (data obtained at 0 mV and 50 μM [Ca2+]free). In addition, hslo channel steady-state activity followed a monotonic function of the POPS concentration in the bilayer (Figure 14). These results also suggest that CHS inhibition of hslo activity discussed above cannot be attributed solely to a dilution of the POPS concentration caused by addition of the sterol to the lipid mixture. Though the addition of 32 mol% CHS to the POPE/POPS (3:1) mixtures causes the PS concentration to drop from 25% to 20% of the total lipid weight, the decrease in P0 the sterol elicits (Figure 13b) is far greater than that expected from the corresponding dilution of POPS (Figure 14).

Figure 15a shows that application of either 50 or 100 mM EtOH fails to significantly increase the NP0 of hslo channels incorporated into pure POPE bilayers
Figure 14. Dilution of POPS in the binary POPE/POPS mixture influences hslo $P_o$ in a concentration dependent manner. $P_o$ values were calculated from hslo channels reconstituted into bilayers with varying weight percentages of POPS in a binary mixture with POPE. Current records were obtained at 0 mV, and $[\text{Ca}^{++}]_{\text{free}} \approx 50 \mu$M on the intracellular side of the bilayer. Data were low pass filtered at 1 kHz and sampled at 10 kHz. $P_o$ values were obtained from continuous recording (Materials and Methods). The number of experiments performed for each condition is shown in parenthesis above each point. *Significantly different from POPE/POPS (3:1, or 25% w/w) values (p<0.05).
(Fig. 15a). The average \( N_p \) during EtOH exposure reached 1.46±0.63 of control \((n=11)\) at 50 mM and 0.60±0.09 of control \((n=4)\) at 100 mM, a concentration near lethal blood levels in naïve mammals (Diamond, 1992). For comparison, potentiation by 50 mM ethanol reached (values compared to control) 5.2±1.5 in 75:25 \((w/w)\) POPE/POPS, 3.3±1.3 in 85:15 \((w/w)\) POPE/POPS, and 1.5±0.6 in 100% POPE, suggesting that the alcohol response may be a monotonic function of POPS concentration in the bilayer.

Next, we determined whether the absence of POPS from the planar bilayer affected CHS inhibition of \( hslo \) channels. Figure 15b demonstrates that, in spite of a large variability in \( P_o \) (as with native \( BK_{Ca} \) channels; Chu et al., 1998), average \( hslo \) activity in POPE+23 mol\% CHS \((p>0.3)\) or POPE+33 mol\% CHS \((p>0.2)\) was not significantly different from that in pure POPE bilayers. Thus, CHS-induced \( hslo \) inhibition, like EtOH-induced \( hslo \) activation, is significantly reduced in a single species POPE bilayer, suggesting that a negatively charged headgroup or other structural requirement associated with the presence of POPS in the bilayer favors CHS and EtOH modulation of \( hslo \) channel function (see Discussion).

**Cholesterol and ethanol target both common and distinct \( hslo \) channel dwelling states.** Modulation of \( hslo \) channel function by CHS and EtOH is characterized by striking similarities: both agents modify channel \( P_o \) with minor, if any, modification of conductance. Furthermore, CHS and EtOH effects on \( hslo \) baseline activity are determined by the amount of POPS in the bilayer (Figure 15). Thus, we considered the possibility that CHS and EtOH actions on \( hslo \) channels are mediated by a single,
Figure 15. Both ethanol and cholesterol fail to markedly modify the activity of hslo channels incorporated into a POPE bilayer. A) EtOH concentrations (50 and 100 mM) that increase hslo channel activity in POPE/POPS bilayers fail to potentiate activity in 100% POPE bilayers. Ratios of NP0 values obtained in the presence and absence of EtOH (left, 50 mM; right, 100 mM) are shown in a scatter graph, where each data point represents an individual bilayer (n). Mean±SEM of data are shown at the top of the graph. The dotted line highlights the point at which NP0 EtOH / NP0 Control=1. The potential at the cis side of the bilayer was set between -20 and +60 mV, and free [Ca2+]i 10 μM. Data were low pass filtered at 1 kHz and sampled at 10 kHz. NP0 values were obtained from continuous recording (Materials and Methods). B) The significant inhibition of hslo activity by CHS in POPE/POPS bilayers is not observed when CHS action is evaluated in POPE bilayers. Scatter graph of hslo NP0 from POPE bilayers in the absence (left) and presence of 23 mol% (middle) or 32 mol% CHS. Data points represent individual bilayers. Mean±SEM of data are shown at the top of the graph. The potential at the cis side of the bilayer was set at 0 mV, and free [Ca2+]i 50 μM. Data were low pass filtered at 1 kHz and sampled at 10 kHz. NP0 values were obtained from continuous recording (Materials and Methods). POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; CHS, cholesterol.
common mechanism. In this case, we might expect channel dwell-time histograms in the presence of each agent to show a mirroring profile of actions.

Dwell times in the absence and presence of 50 mM EtOH were evaluated in several POPE/POPS (3:1) bilayers (V=0-20 mV; [Ca$^{++}$]$_{free}$=15 µM; n=3). In both the absence and presence of EtOH, the open times distribution could be well fitted with a double exponential (Figure 16a), indicating the existence of at least two open states. Although 50 mM EtOH characteristically increases channel NPo to ~5-fold of control values, on average, it only slightly increased the duration of short and long openings. These drug-induced changes in open channel populations result only in a minor change in mean open time. Thus, a major increase in hslo steady state activity induced by acute EtOH could be obtained in the absence of a significant increase in channel mean open time.

The closed times distribution was also well fitted with a double exponential function, in both the absence and presence of EtOH, (Figure 16b), indicating the existence of at least two closed states. Two actions of EtOH are evident: a decrease in the average duration of long closures, and a marked shift in the closed channel population from long to brief events. EtOH-induced changes in the closed times distribution result in a significant change in mean closed time (~60% of control), the major contributor to EtOH-induced increase in channel P0. In summary, EtOH markedly increases hslo steady-state activity by producing a marked reduction in the average duration of channel long closures and their relative contribution to the total time spent in closed states, without causing a major change in the channel mean open time.
Figure 16. Representative dwell-times distributions of *hslo* channel activity reconstituted into POPE/POPS 3:1 (w/w) bilayers in the absence (top) and presence (bottom) of 50 mM EtOH, which show the targeting of open (A) and closed (B) times by the drug, that lead to potentiation of channel activity. A) Open time distributions in both absence and presence of EtOH were well fitted with a double exponential. EtOH slightly increased the duration of short openings while slightly shifting the channel population from long to short openings; B) Closed time distributions in both absence and presence of EtOH were also well fitted with a double exponential. EtOH decreased the duration of long closures and markedly shifted the channel population from long to short closures. The potential at the cis side of the bilayer was set between 0 and 20 mV, and free [Ca\(^{++}\)]\(_{ic}\)≈15 μM. In both A) and B), each panel shows the total number of events (N), the duration of each particular component (t, in msec), and the relative contribution of each component to the total fit (in parentheses). The solid line represents the composite fit. Data were low pass filtered at 3 kHz and sampled at 10 kHz. The following are mean±SEM values (n=3) for the time constants (msec) and the fractional contribution of each component to the total fit respectively: control open states, \(\tau_{fast}=0.768±0.142\) and 0.150±0.016, \(\tau_{slow}=7.917±0.962\) and 0.850±0.016; open states in EtOH, \(\tau_{fast}=0.963±0.178\) and 0.176±0.014, \(\tau_{slow}=8.643±1.572\) and 0.824±0.014; control closed states, \(\tau_{fast}=0.712±0.178\) and 0.206±0.008, \(\tau_{slow}=218.671±8.671\) and 0.794±0.008; closed states in EtOH, \(\tau_{fast}=0.588±0.015\) and 0.335±0.046, \(\tau_{slow}=151.996±7.811\) and 0.659±0.049.
Were CHS and EtOH having opposite effects on channel $P_\circ$ solely by targeting common kinetic states in opposite fashion, we might expect a profile of changes in open and closed times distributions by CHS mirroring those caused by EtOH. This complementarity was indeed present, but each of these agents also had unique effects not mirrored by the other. The dwell time data for $hslo$ channels in POPE/POPS (3:1) bilayers with increasing amounts of CHS (n=2–8 for each CHS concentration) were obtained under identical conditions of voltage (0 mV) and $[\text{Ca}^{2+}]_{\text{free}}$ (50 $\mu$M). The closed times distributions in the presence and absence of CHS could be well fitted with two exponentials in the representative example shown (Figure 17b), which is particularly useful for a comparison with the closed time distribution in the presence of EtOH (Figure 16b). These data demonstrate that CHS increases the average duration of longer closures and shifts the closed channel population from short to longer closures, these two changes being a mirror of EtOH actions that lead to channel activation. However, CHS also increases the average duration of short closures, an action not mirrored by EtOH. In addition, representative open times distributions (Figure 17a), fitted by double exponential functions, indicate that CHS at all concentrations tested decreased the average duration of long openings, a dwell state basically unmodified by EtOH. Figure 17a also shows that CHS produces a minor shift from long to short openings, being another contributor to the decrease in mean open time induced by CHS. In summary, overall antagonism between CHS and EtOH on $hslo$ steady-state activity results from the targeting of both common and distinct channel dwelling states by these modulators, probably reflecting their common and distinct sites of action (see Discussion).
Figure 17. Representative dwell-times distributions of hslo channel activity reconstituted into POPE/POPS 3:1 (w/w) bilayers in the absence (top panels) and presence of increasing amounts of cholesterol. The potential at the cis side of the bilayer was set at 0 mV, and free [Ca\(^{++}\)]\(_{ic}\)=50 μM. A) Open time distributions in both absence and presence of CHS were well fitted with a double exponential. Increases in CHS content up to 23 mol% progressively decrease the average duration of long openings; B) Closed time distributions in both absence and presence of CHS were also well fitted with
a double exponential function. Increases in CHS content (11-49 mol%) progressively shift the channel population from short to long closures, and increase the duration of long closures. In both A) and B), each panel shows the total number of events (N), the duration of each particular component (t, in msec), and the relative contribution of each component to the total fit (in parentheses). The solid line represents the composite fit. Data were low pass filtered at 3 kHz and sampled at 10 kHz. The average values for the time constants (msec) and the fractional contribution of each component to the total fit for the open states were: POPE/POPS, $\tau_{\text{fast}}=1.61\pm0.393$ and $0.139\pm0.041$, $\tau_{\text{slow}}=11.765\pm2.111$ and $0.861\pm0.041$ (n=8); POPE/POPS+10 mol% CHS, $\tau_{\text{fast}}=1.557\pm0.356$ and $0.185\pm0.09$, $\tau_{\text{slow}}=5.773\pm0.333$ and $0.815\pm0.09$ (n=5); POPE/POPS+23 mol% CHS, $\tau_{\text{fast}}=1.242\pm0.309$ and $0.239\pm0.073$, $\tau_{\text{slow}}=4.194\pm0.77$ and $0.704\pm0.108$ (n=7); POPE/POPS+32 mol% CHS, $\tau_{\text{fast}}=1.046\pm0.218$ and $0.22\pm0.079$, $\tau_{\text{slow}}=4.628\pm0.246$ and $0.779\pm0.079$ (n=4); POPE/POPS+49 mol% CHS, $\tau_{\text{fast}}=1.066$ and $0.28$, $\tau_{\text{slow}}=6.981$ and $0.72$ (n=2). The average values for the time constants (msec) and fractional contribution of each component of the distribution to the total fit for the closed states were: POPE/POPS, $\tau_{\text{fast}}=0.436\pm0.094$ and $0.835\pm0.033$, $\tau_{\text{slow}}=12.994\pm5.904$ and $0.165\pm0.033$ (n=8); POPE/POPS+10 mol% CHS, $\tau_{\text{fast}}=0.694\pm0.053$ and $0.671\pm0.07$, $\tau_{\text{slow}}=19.152\pm4.341$ and $0.329\pm0.07$ (n=5); POPE/POPS+23 mol% CHS, $\tau_{\text{fast}}=0.587\pm0.113$ and $0.533\pm0.078$, $\tau_{\text{slow}}=21.022\pm4.11$ and $0.467\pm0.077$ (n=7); POPE/POPS+32 mol% CHS, $\tau_{\text{fast}}=1.155\pm0.126$ and $0.566\pm0.079$, $\tau_{\text{slow}}=42.971\pm13.052$ and $0.434\pm0.079$ (n=4); POPE/POPS+49 mol% CHS, $\tau_{\text{fast}}=1.462$ and $0.67$, $\tau_{\text{slow}}=146.06$ and $0.33$ (n=2).
Discussion

The role of the lipid environment in the function of embedded ion channel proteins and their drug sensitivity is difficult to assess in complex biological systems, but is more approachable in reduced preparations such as with cloned channels incorporated into planar lipid bilayers. Our data demonstrate the feasibility of this approach, since hslo channels in POPE and POPE/POPS bilayers retain basic BKCa channel characteristics and respond to the change in bulk phospholipid composition. Of course, this reductionist approach ignores many of the potential interactions present in a rich, heterogeneous natural membrane. However, the model used successfully addresses the specific questions that are being asked.

Modulation of BKCa activity by ethanol and cholesterol is reduced in POPE bilayers.

We demonstrate that both CHS and EtOH modulation of basal hslo activity are dramatically impaired in the absence of POPS in the bilayer. The fact that these agents modulate hslo channel activity in POPE/POPS, but fail to do so in pure POPE bilayers could be attributed to the loss of headgroup negative charge, differing headgroup size, and/or altered headgroup interactions. Neutral PE bilayers have a high propensity to transition from the lamellar phase into the HII (inverted hexagonal) phase (Figure 22), a transition directly attenuated by negative membrane surface charge carried by PS (Lewis and McElhaney, 2000). Though this transition for POPE generally occurs at higher temperatures than those employed in this study (70°C; Pare and Lafleur, 1998), we cannot rule out that such a tendency may mask or alter the actions of CHS or EtOH under the exact conditions employed in our system. Both PE and CHS are nonlamellar phase-
preferring lipids, which can presumably increase curvature stress when incorporated into a lamellar bilayer (Stubbs and Slater, 1996; Lundbaek et al., 1996). It is possible that a pure POPE bilayer with a high initial degree of curvature stress (Figure 23) masks sterol modulation of this parameter. The inability of CHS to modify hSlo function when added to POPE bilayers might also reflect a relatively low lateral miscibility of CHS in pure PE (McMullen et al., 1999), perhaps alleviated by the headgroup structure and charge in the POPE/POPS mixture.

**Increases in membrane cholesterol, which occur in animal models of chronic ethanol exposure, antagonize ethanol actions on BKCa channels.**

In both cell culture and animal models of chronic EtOH exposure, alterations in both content (Chin et al., 1978; Omodeo-Sale et al., 1995) and distribution (Wood et al., 1990a) of membrane CHS have been reported. These alterations might represent a compensatory response (i.e., “tolerance”) to counteract the effects of EtOH on relevant targets, such as defined ion channel populations. Here, we demonstrate that increases in the CHS content of POPE/POPS bilayers, indeed, reduce EtOH potentiation of hSlo channel P₀.

Increases in bilayer/membrane CHS content diminish the lipid/membrane partition coefficient of a variety of small anesthetics, such as halothane (Lechleiter et al., 1986), uncharged pentobarbitone (Miller and Yu, 1977), and benzyl alcohol (Colley and Metcalfe, 1972). Isothermal titration calorimetry data show that EtOH partitioning into phosphatidylcholine (PC) liposomes is also reduced by CHS, when present in the bilayer at concentrations >10 mol% (Trandum et al., 2000). Consistent with these findings,
Figure 12 demonstrates that CHS effects on EtOH sensitivity of *hslo* channels is largely absent at 10 mol% CHS, but evident at concentrations above 23 mol%.

The effect of CHS on EtOH partitioning may be explained by bilayer phase behavior. Isothermal titration calorimetry (Trandum *et al.*, 1999) and computer stimulation (Jorgensen *et al.*, 1993) studies strongly suggest that EtOH preferentially partitions into bilayers at the interfaces between the gel and the liquid crystalline domains that form as the bilayer approaches the transition temperature. High bilayer CHS concentrations abolish the gel to liquid crystalline transition, causing the bilayer to exist in a liquid-ordered state (Trandum *et al.*, 2000). The resulting disappearance of the gel/liquid crystalline interfaces preferentially targeted by EtOH would serve to decrease its partitioning into the membrane. In fact, X-ray diffraction studies of POPE/POPS mixtures suggest that at 25°C, multilayer samples exist as a combination of both gel and liquid crystalline phases (Chang *et al.*, 1995b). The coexistence of these lateral domains would support the partitioning of EtOH into this lipid mixture. We show here that channels are sensitive to EtOH in POPE/POPS bilayers (Figure 9, Figure 11). However, in bilayers containing >20 mol% CHS which lack these domain interfaces (Chang *et al.*, 1995b), EtOH potentiation of *hslo* activity is markedly reduced (Figure 12). Thus, our data are consistent with a CHS-induced reduction of EtOH partitioning in the bilayer. In our system, however, the hydrocarbon interior of the bilayer should contain contaminant decane in equilibrium with that in the torus that surrounds the bilayer (Gruen, 1981), so we cannot rule out some contribution of this solvent to the phase behavior of the bilayers employed in our study. The manipulation of parameters like temperature and acyl chain...
saturation will yield further insight into the importance of bilayer phase behavior on BKCa ethanol sensitivity.

Further evidence that CHS reduces EtOH interaction with the membrane comes from NMR spectroscopy data demonstrating that EtOH resides at the lipid-water interface in phospholipid bilayers. The carbonyl groups in the glycerol backbone are specifically favored hydrogen bonding sites for EtOH (Barry and Gawrisch, 1994), this binding being decreased by increasing amounts of CHS (Barry and Gawrisch, 1995). Since CHS interacts with PC at the same carbonyl groups in the glycerol backbone (Worcester and Franks, 1976), this was interpreted as CHS directly competing for EtOH’s favored binding sites (Barry and Gawrisch, 1995). CHS location at the phospholipid carbonyl groups would also increase the packing density of the phospholipids and antagonize the increase in acyl chain motion (“disordering”) introduced by EtOH (Sun and Sun, 1985), which may contribute to functional antagonism on hslo kinetics (see below).

Comparison of ethanol and cholesterol actions on BKCa kinetics and bilayer physical properties.

Apart from effects on EtOH partitioning, CHS may directly antagonize the action of the drug on the bilayer or the hslo channel itself. If CHS and EtOH act through a single, common mechanism, we might expect them to exert reciprocal actions on common dwell states of the channel. Indeed, EtOH and CHS produce a mirrored shift between the long and short channel closed states and have opposite actions on the mean duration of long closures. This suggests that CHS and EtOH share a common target on
the channel protein or in the phospholipid bilayer, which is important in determining the stability of the channel closed state(s). A similar increase in the average duration of native BKCa channel long closures following CHS enrichment of myocyte membranes has been reported (Bolotina et al., 1989). Channel P0 was approximately halved, coincident with a similar decrease in the rotational diffusion coefficient of DPH (Bolotina et al., 1989), indicating that a reduction in acyl chain order accompanies the reduction in channel activity. Spin-labeling experiments also demonstrate that CHS increases while EtOH decreases bilayer order (Chin and Goldstein, 1981). Interestingly, the magnitude of CHS and EtOH effects on multilayer PC vesicle order are similar to those in brain synaptosomal membranes (Chin and Goldstein, 1981). Thus, we postulate that opposite actions of CHS and EtOH on acyl chain order may underlie or, at least, contribute to CHS and EtOH opposing effects on common channel dwell states, such as the long closed state.

In addition to their common modulation of channel long closures, CHS and EtOH exhibit individual effects on channel dwell times, which contribute to their opposite actions on P0. These distinct effects on channel dwell states may represent independent actions of these modulators on specific bilayer characteristics that modify channel function. For example, EtOH increases the rate of phospholipid desorption, displacing water from the hydrogen-bonded network of water molecules in the hydration layer, whereas CHS has little, if any, effect on phospholipid desorption (Slater et al., 1993). Changes in phospholipid desorption alter not only lipid-lipid interactions, but also protein-lipid interactions, with eventual modification of ion channel function.
Interestingly, EtOH desorption is more marked in PS than PE (Slater et al., 1993), consistent with the EtOH activation of hslo channels (Figures 11 and 15).

Monolayer or bilayer properties modified by CHS, but not EtOH, include a broadening and eventual elimination of the gel-to-liquid crystalline phase transition (Figure 2), a decrease in the cross sectional area occupied by the phospholipid in the liquid-crystalline state (Figure 1), increases in both bilayer thickness and mechanical strength, and increases in the lateral stress (Figure 23) and stiffness of the phospholipid monolayer or bilayer in the physiologically relevant fluid phase (McMullen et al., 1999; Nielsen et al., 1999). In particular, changes in lateral stress and bilayer stiffness have been causally related to modification of ion channel function. Cholesterol and other compounds promoting negative monolayer curvature increase stiffness and decrease channel activity, while compounds promoting positive monolayer curvature have opposite effects on both stiffness and channel activity (Lundbaek et al., 1996; Bezrukov et al., 1998). Furthermore, CHS inhibition of native BKCa channels in PE/PS bilayers has been linked to an increase in bilayer lateral stress caused by the presence of the sterol (Chang et al., 1995b). A major consequence of increases in lateral stress is a reduction in the activation energy for the transition from open to closed state(s). This reduces the average duration of long openings, as we report here (Figure 17a). Thus, we postulate that the distinct decrease in the average duration of long openings observed with CHS, an effect not mirrored by EtOH, may be related to the increase in lateral stress caused by the sterol.

Summary.
In our demonstration of CHS blunting of alcohol potentiation of hslo activity we used 50 mM EtOH, close to legal intoxication (~20 mM) and below lethal blood levels in naïve subjects (> 90 mM), and a range of CHS content similar to that found in cell membranes (5.6-44 mol%). Thus, modification of hslo function by EtOH may depend on the membrane CHS content where the channel resides. Membrane CHS content and/or distribution might contribute to differential EtOH sensitivity of BKCa in different cell types and in similar channel subtypes from different neuronal domains, as in supraoptic neurons (Dopico et al., 1999). Present results might also help to explain recent findings that rats chronically fed with EtOH display not only reduced BKCa current density in neurohypophysial terminals, but also reduced BKCa sensitivity to acute EtOH (Knott et al., 2002).

Our findings suggest that manipulation of membrane lipid composition may represent a mechanism for plasticity responsible for alteration of channel basal Po as well as sensitivity to small amphiphiles such as EtOH. This hypothesis becomes particularly attractive when coupled with data demonstrating alterations in lipid composition after EtOH exposure, and more generally with the emerging theme of lipid domains. Here, we demonstrate that alcohol action on the activity of a human neuronal ion channel depends on the lipid environment of the channel protein.
Reference List


CHAPTER III
ROLE OF SURFACE CHARGE, LIPID MOLECULAR SHAPE, AND ACYL CHAIN SATURATION IN THE BASAL ACTIVITY AND ETHANOL SENSITIVITY OF RECONSTITUTED HSLO CHANNELS

Introduction

Membrane lipids differ widely in their structure, and in the physical properties they impart on the bilayer and its transmembrane channels (Andersen et al., 1999; Barrantes, 2002). The large conductance Ca\(^{++}\)-activated K\(^{+}\) (BK\(_{Ca}\)) channel has served as a model for assessing the influence of lipid environment on channel function. Alterations in cholesterol (CHS) levels (Chang et al., 1995b; Figure 13), membrane surface charge (Moczydlowski et al., 1985; Turnheim et al., 1999; Figure 14), and headgroup structure (Chang et al., 1995a) can influence the basal activity of both native and cloned BK\(_{Ca}\) channels reconstituted into planar bilayers. Through the rapid efflux of K\(^{+}\) in response to membrane depolarization and increases in intracellular free Ca\(^{++}\), these channels regulate cellular excitability and neurotransmitter release. The modulation of BK\(_{Ca}\) function by membrane lipid composition is of particular interest given the non-random leaflet (Devaux and Zachowski, 1994) and lateral domain (Welti and Glaser, 1994) organization of the plasma membrane. Moreover, membrane lipid composition is tightly regulated (Brown and Goldstein, 1999; Thewke et al., 2000), perhaps as a homeostatic mechanism to maintain optimal membrane function. These findings raise the possibility that local differences in lipid composition and physical properties may arise, which regulate the function of BK\(_{Ca}\) channels.
EtOH is an amphiphilic molecule that potentiates BK$_{Ca}$ channels when applied acutely in native membranes (Dopico et al., 1996; Knott et al., 2002), ripped-off patches (Dopico et al., 1998), and in the planar lipid bilayer (Chu et al., 1998; Figure 9 and 11). BK$_{Ca}$ channels isolated from the neurohypophysis of rats chronically exposed to EtOH, however, exhibit a reduction in sensitivity to the drug (Knott et al., 2002). Interestingly, animal models of chronic EtOH exposure also show alterations in the amount (Chin et al., 1978; Omodeo-Sale et al., 1995) or distribution (Wood et al., 1989, 1990a) of CHS, as well as changes in the acyl chain profile and headgroup composition of the plasma membrane from a variety of tissues (Swann, 1987). Since the basal function and EtOH potentiation of BK$_{Ca}$ persist in a planar bilayer system, composed of cloned human BK$_{Ca}$ (hslo) channels reconstituted into 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE)/ 1-palmitoyl-2-oleoyl-phosphatidylserine (POPS) mixtures, the influence of such changes in membrane lipid composition can be determined. We have previously shown that the potentiation of hslo by EtOH is significantly reduced in both pure POPE bilayers (Figure 15) and POPE/POPS (3:1) mixtures with high (>23 mol%) cholesterol (CHS) (Figure 12) concentrations. In addition, the ability of CHS to inhibit basal hslo $P_o$ is reduced in the pure POPE membrane relative to the POPE/POPS (3:1) mixture (Figure 15). Here, we begin to explore the contribution of lipid physical properties such as negative surface charge, lipid molecular shape, and acyl chain saturation in the regulation of hslo basal activity and EtOH sensitivity.

The phosphatidylserine (PS) headgroup carries a net negative charge at physiological pH. BK$_{Ca}$ channels reconstituted into negatively charged planar bilayers
exhibit higher open probability ($P_0$) values relative to those in neutral bilayers, perhaps as a result of relative increases in the Ca$^{++}$ concentration at the intracellular face of the membrane (Moczydlowski et al., 1985; Turnheim et al., 1999). The decreased efficacy of both EtOH and CHS in the modulation of channels in POPE bilayers, versus POPE/POPS (3:1) mixtures, suggests that negative charge or PS headgroup structure could be required in the mechanism of action of these agents on $hslo$ channels in our system.

A second interpretation stems from the molecular shape of phosphatidylethanolamine (PE). PE molecules have a small polar headgroup cross-section relative to their hydrophobic region, yielding a molecular “cone” shape and a preference for nonlamellar phases (Figure 22) that creates a membrane curvature stress (Gruner, 1985; Israelachvili et al., 1980). CHS exerts similar effects on membranes (Lundbaek et al., 1996; Figure 22), and neither pure POPE nor CHS-containing (>23 mol%) bilayers support EtOH modulation of reconstituted $hslo$ channels (Figure 12 and 15). Furthermore, the nonlamellar propensity of POPE is attenuated by negatively charged POPS (Epand and Bottega, 1988; Lewis and McElhaney, 2000), and these mixtures support drug action (Chu et al., 1998; Figure 9 and 11). This correlation suggests that “cone” shape nonlamellar lipids may antagonist the actions of EtOH on $hslo$ channels, perhaps through increases in curvature stress. Furthermore, if CHS inhibition of $hslo$ $P_0$ depends upon its molecular “cone” shape, its actions may be masked in pure POPE membranes with a greater initial curvature stress relative to the POPE/POPS (3:1) mixtures that support sterol inhibition.
In addition to surface charge and curvature stress, other lipid physical properties may contribute to modulation of basal activity and EtOH sensitivity of hslo channels in our system. CHS increases the order of phospholipid acyl chains above the main phase transition of the membrane, and antagonizes the disordering actions of EtOH on acyl chains (Chin and Goldstein, 1981). This is coincident with sterol inhibition of basal hslo Po and antagonism of EtOH action on reconstituted hslo channels in POPE/POPS (3:1) mixtures (Figure 12 and 13). CHS addition to POPE/POPS (3:1) also creates a ternary mixture with a more diverse set of interactions between headgroups. It is possible that the complexity of the headgroup interactions in a ternary mixture may contribute to the differences in basal activity and EtOH sensitivity from the POPE/POPS (3:1) mixture.

Here, we test the ability of EtOH and CHS to modulate channel function in neutral POPE/1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) bilayers. POPC is uncharged, but it has a "cylindrical" molecular shape with proportional polar and nonpolar moieties. It strongly prefers lamellar phases, and in a mixture with POPE, it can counteract the ability of POPE to form nonlamellar phases (Epand and Bottega, 1988). The role of bilayer surface charge can, therefore, be separated from any impact on protein function caused by the enhanced nonlamellar tendencies of the pure POPE membrane relative to the POPE/POPS (3:1) mixture, in regards to hslo modulation by EtOH and CHS. We next assess the ability of 20 mol% POPC to modulate both basal activity and EtOH sensitivity of reconstituted hslo channels in the POPE/POPS (3:1) background. Like the addition of CHS, this substitution creates a ternary lipid mixture with a more complex set of headgroup interactions. Based on the molecular shape of POPC and CHS,
however, these molecules do not influence curvature stress in the same manner. This allows separation of the complexity of a ternary lipid mixture from the physical properties of the CHS molecule itself. Finally, we test a PE/PS mixture containing only monounsaturated oleic acid side chains, predicted to decrease acyl chain order, for the basal activity and drug response of reconstituted hslo channels to assess any correlation between bilayer order and channel function.

**Materials and Methods**

**HEK 293 membrane preparation.** HEK-293 membrane fragments were isolated using a protocol for COS cells (Sun et al., 1994), modified slightly. Briefly, HEK 293 cells stably transfected with hslo cDNA (a gift from Dr. P. Ahring, NeuroSearch A/S, Denmark) were grown to confluence, pelleted, and resuspended on ice in 10 ml of buffer (mM): 30 KCl, 2 MgCl₂, 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 5 ethylene glycol-bis(β-aminoethyl ether)-N,N’,N’’,N’’-tetraacetic acid (EGTA); pH 7.2. The cell suspension was forced through a 27-gauge needle 4 times, and sonicated at 30% maximum power for 30 sec, twice. The suspension was layered on a 20-38% sucrose density gradient (in 20 mM MOPS, pH 7.1) and centrifuged at 25,000 rpm for 60 min at 4°C. The band at the 20%-38% interface was collected with a syringe, diluted with bidistilled H₂O, and centrifuged in a 50.2 Ti rotor at 45,000 rpm for 60 min at 4°C. The resulting pellet was resuspended in 200 μl of buffer (mM): 250 sucrose, 10 HEPES; pH 7.3. Aliquots were stored at -80°C.

**Electrophysiology.** Channels were incorporated by dropping 0.5 μl of the membrane preparation onto preformed bilayers cast from mixtures of POPE, POPS, POPC and CHS.
Lipids were dried under N2 gas, and resuspended in decane, for a final lipid concentration of 25 mg/ml. Bilayers were formed by painting the lipid mixture across a 100 μM hole formed in a plastic coverslip (Wonderlin et al., 1990). Capacitance was monitored by the capacitive 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 their Ca²⁺-sensor facing the cis chamber were studied. Solutions consisted of (mM): cis, 300 KCl, 10 HEPES, 1.10 N-(2-hydroxyethyl) ethylene-diaminetriacetic acid (HEDTA) ([Ca⁺⁺]ₜₐᵢₙₜ 55 μM), 1.05 CaCl₂, pH 7.2, and trans, 30 KCl, 10 HEPES, 0.1 HEDTA, pH 7.2. CHS action on channel function was studied at 55 μM [Ca⁺⁺]ₜₐᵢₜ, which promotes high channel activity, from which the inhibitory effects of CHS can be effectively quantitated. EtOH sensitivity was tested at 10 μM [Ca⁺⁺]ₜₐᵢₜ, which yields lower activity and prevents a "ceiling effect" when measuring EtOH potentiation. The magnitude of EtOH activation of slo activity is independent of voltage within the range studied here (Dopico et al., 1996). [Ca⁺⁺]ₜₐᵢₜ in the cis chamber was adjusted using aliquots from a 1 M stock solution of HEDTA (pH 7.2). [Ca⁺⁺]ₜₐᵢₜ values given are nominal, calculated using the Max Chelator Sliders program (C. Patton, Stanford University).

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 either 3 (for dwell
time analysis) or 1 kHz (for display and NP, determination) 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 6.0.2 (Axon Instr., 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). N was monitored pre- and post-EtOH by stepping to positive potentials to maximize P. Experiments showing an increase in N after EtOH addition were discarded. NP, was determined from periods of at least 20 sec of continuous recording. Data are shown as mean±S.E.M. The significance of the difference between means was determined by Student’s t tests.

**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, DOPE, DOPS, POPC, and CHS from Avanti Polar Lipids (Alabaster, AL).

**Results**

We have shown recently that the modulation of hslo channel activity by EtOH and CHS is reduced in a POPE background, relative to a POPE/POPS (3:1) mixture (Figure 12). POPE bilayers lack negative surface charge, but may also exhibit increased curvature stress due to the “cone” shape and resulting nonlamellar tendency of the PE headgroup. Here, we set out to distinguish between these two possibilities by employing a POPE/POPC (3:1) mixture that dissociates the lack of negative surface charge from the properties of the pure POPE membrane. We first demonstrate that, like channels in
POPE membranes, *hslo* channels reconstituted into uncharged POPE/POPC (3:1) bilayers exhibit lower $P_o$ and $g$ values than channels in charged POPE/POPS (3:1) membranes, under identical conditions (0 mV, $[\text{Ca}^{++}]_{\text{free}} \approx 50$ $\mu$M, KCl gradient 300 mM/30 mM i/o):

POPE/POPS (3:1) $P_o = 0.787 \pm 0.058$ (n= 8), $g = 330 \pm 6$ pS (n= 16) vs. POPE/POPC (3:1) $0.236 \pm 0.067$ (n= 12), $g = 304 \pm 10$ pS (n= 17) (p < 0.05). This result is consistent with previous findings addressing $\text{BK}_{\text{Ca}}$ channel activity in charged and uncharged bilayers (Moczydlowski et al., 1985; Tumheim et al., 1999; Park et al., 2003).

**CHS and EtOH modulation of *hslo* channels reconstituted into POPE/POPC (3:1) bilayers.** Figure 18a shows representative traces of the activity of single *hslo* channels recorded under identical conditions (+30 mV, $[\text{Ca}^{++}]_{\text{free}} \approx 50$ $\mu$M, KCl gradient 300 mM/30 mM i/o) in POPE/POPC (3:1) bilayers lacking (left) and containing (right) CHS. In this background, 23 mol% CHS is able to reduce $hslo$ $P_o$ nearly 80% (Figure 18b; POPE/POPC (3:1) $P_o = 0.406 \pm 0.077$ (n= 12) vs. POPE/POPC/23 mol% CHS $P_o = 0.091 \pm 0.069$ (n=4) (p < 0.05). Slope conductance, as in the POPE/POPS (3:1) background (Chapter II), appears unaffected (POPE/POPC $g = 304 \pm 10$ pS (n= 18), vs.

POPE/POPC/23 mol% CHS $g = 312 \pm 18$ pS (n= 5). This result suggests CHS modulation of *hslo* channel activity is independent of both negative surface charge and PS headgroup structure itself.

We have shown that EtOH action on hslo channels is also reduced when reconstituted into uncharged POPE bilayers, versus that seen in POPE/POPS (3:1) membranes. Channels in these uncharged POPE membranes are not potentiated by the
Figure 18. Cholesterol can inhibit hsto activity in uncharged POPE/POPC mixtures. (A) Representative traces from single channels reconstituted into POPE/POPC (3:1) membranes (top) and POPE/POPC/23 mol% CHS (bottom) bilayers. Records were obtained at +30 mV, with ~50 μM [Ca$^{++}$]$_{\text{Free}}$ on the intracellular side of the bilayer. Data was low-pass filtered at 1 kHz, and sampled at 10 kHz. (B) Hsto P$_o$ is significantly reduced by 23 mol% CHS in an uncharged POPE/POPC (3:1) background (*, p < 0.05).
drug at both 50 mM (8/11 channels) and 100 mM (4/4 channels) concentrations (Figure 15). Figure 19a contain representative traces of the same hslo channel in a POPE/POPC (3:1) bilayer before (above) and after (below) the addition of 50 mM EtOH to the upper chamber. As in the POPE/POPS (3:1) mixture, the drug potentiated channel activity in 6/7 of experiments when studied in the uncharged POPE/POPC (3:1) mixture. The magnitude of the response to 50 mM EtOH was smaller, though not statistically different, than observed in POPE/POPS (3:1) mixtures (2.41X vs. 5.19X, respectively) (p≈0.1, Student’s t-test) (Figure 19b). For comparison, the data obtained previously in POPE membranes (from Figure 15) are shown. This suggests that, as for CHS modulation of hslo channels, negative surface charge and PS headgroup structure are not necessary to support EtOH action on channel activity.

**Modification of hslo function by a ternary headgroup composition.** The addition of CHS to POPE/POPS (3:1) bilayers results in a significant decrease in both the basal activity and EtOH sensitivity of reconstituted hslo channels (Figure 12 and 13). The addition of CHS would be expected to increase curvature stress (Chang et al., 1995b; Lundbaek et al., 1996), increase acyl chain order (Ohvo-Rekila et al., 2002), and create a ternary mixture with a more complex set of lipid interactions. It is unclear which of these attributes, if any, may explain the influence of the sterol on hslo function. In contrast, a ternary mixture created by adding POPC to the POPE/POPS (3:1) combination, while yielding a comparable greater variety of lipid interactions, should not influence acyl chain order or curvature stress in the same manner as CHS. In spite of this, Figure 20a
Figure 19. EtOH activates hslo channels in uncharged POPE/POPC mixtures. (A) Representative traces of hslo channel activity in a POPE/POPC bilayer before and after the addition of 50 mM EtOH. Records were obtained at +10 mV, with ~10 μM [Ca^{2+}]_{free} on the intracellular side of the bilayer. Data was low-pass filtered at 1 kHz, and sampled at 10 kHz. (B) EtOH consistently activates hSlo channels (6/7 cases) in POPE/POPC (3:1) bilayers, as in POPE/POPS (3:1) membranes (N.S., p ≈ 0.1). For comparison, the data obtained previously in uncharged POPE mixtures is shown. Each point represents a single experiment, and arrows indicate overlapping values. The n for each condition is shown on the plot, and the Mean (S.E.M.) is displayed above each column (*, p < 0.05).
Figure 20. POPC (20 mol%) added to the POPE/POPS (3:1) mixture can reduce the basal activity of reconstituted hslo channels, but cannot significantly reduce EtOH sensitivity. (A) Open probability of hslo channels reconstituted into POPE/POPS (3:1), POPE/POPS (3:1) + 23 mol% CHS (Crowley et al, 2003), and POPE/POPS (3:1) + 20 mol% POPC. Records were obtained at 0 mV, with ~50 μM [Ca$^{++}$]$_{Free}$ on the intracellular side of the bilayer. Data was low-pass filtered at 1 kHz, and sampled at 10 kHz. (B) NP$_o$ EtOH/NP$_o$ Control values for hslo channels in the same mixtures. Records were obtained between -10 and +30 mV, with ~10 μM [Ca$^{++}$]$_{Free}$ on the intracellular side of the bilayer. Each point represents a single experiment, and arrows indicate overlapping values. The n for each condition is shown on the plot, and the Mean (S.E.M.) is displayed above each column (*, p < 0.05).
demonstrates that the addition of 20 mol% POPC to the POPE/POPS (3:1) mixture can reduce the basal $P_o$ of $hslo$ in a manner similar to a roughly equivalent amount (23 mol%) of CHS (Figure 13b), when recorded under the same conditions (0 mV, $[Ca^{++}]_{\text{Free}} \approx 50 \mu M$, KCl gradient 300 mM/30 mM i/o). However, the addition of POPC doesn’t antagonize the potentiation of the channel by 50 mM EtOH (Figure 20b). Channels are strongly activated by 50 mM EtOH in 3/8 cases, not significantly changed in 4/8 cases, and inhibited in 1/8 cases, yielding an average fold increase of $2.29 \pm 0.79$, that is not statistically different from POPE/POPS (3:1) ($p \approx 0.1$). These observations suggest that modulation of basal $hslo$ $P_o$ can occur without large predicted alterations in acyl chain order, or increases in curvature stress. However, significant changes in EtOH sensitivity appear to require modulation of bilayer properties beyond the increase in headgroup diversity introduced here.

The influence of acyl chain order on the activity and EtOH sensitivity of reconstituted $hslo$ channels. To address the importance of acyl chain order, we next studied a 3:1 PE/PS mixture containing mono-unsaturated oleic side chains at both the sn-1 and sn-2 position (DOPE/DOPS). Bilayers cast from this mixture will be less ordered than the corresponding POPE/POPS (3:1) bilayer due to the lack of saturated chains in the hydrocarbon interior. Figure 21a demonstrates that, relative to POPE/POPS (3:1), the basal activity of $hslo$ channels is significantly decreased in the DOPE/DOPS (3:1) mixture, under identical conditions (0 mV, $[Ca^{++}]_{\text{Free}} \approx 50 \mu M$, KCl gradient 300 mM/30 mM i/o). However, acyl chain order does not correlate directly with $BK_{Ca}$.
Figure 21. Hslo channels in DOPE/DOPS (3:1) bilayers containing only mono-unsaturated acyl chains exhibit reduced basal $P_o$, but maintain sensitivity to 50 mM EtOH. (A) Open probability values for channels in POPE/POPS (3:1) and DOPE/DOPS (3:1) bilayers. Records were obtained at 0 mV, with 50 μM $[Ca^{++}]_{free}$ on the intracellular side of the bilayer. Data was low-pass filtered at 1 kHz, and sampled at 10 kHz. The n (in parenthesis) is shown for each data set (*, p < 0.05). (B) Scatter plot of NPo EtOH/NPo Control values for channels in POPE/POPS (3:1) and DOPE/DOPS (3:1) bilayers. Records were obtained with 10 μM $[Ca^{++}]_{free}$ on the intracellular side of the bilayer. Each point represents a single experiment, and arrows indicate overlapping values. The n for each condition is shown on the plot, and the Mean (S.E.M.) is displayed above each column.
channel $P_o$, since manipulations expected to increase (POPE/POPS (3:1) +23 mol% CHS) or decrease (DOPE/DOPS (3:1)) bilayer order both elicit decreases in the basal activity of reconstituted $hso$ channels.

If reductions in membrane order caused by EtOH (Chin and Goldstein, 1981) were a determining factor of EtOH action on BK$_{Ca}$ channels, we might expect a decrease in chain order to increase channel activity, as EtOH does. As shown above, this is not the case. Through the same logic, we might expect decreases in membrane order to enhance the actions of the drug on BK$_{Ca}$ channels. However, Figure 21b demonstrates that $hso$ channels in DOPE/DOPS (3:1) bilayers are potentiated by 50 mM EtOH in only 4/10 cases, unchanged in 3/10 cases, and inhibited in 3/10 cases. Overall, the fold increase in open probability elicited by the drug was 2.93±1.13, which was not statistically different from that observed in POPE/POPS (3:1) bilayers ($p=0.25$). These results suggest a reduction in bilayer order is unlikely to explain the actions of the drug on the gating of BK$_{Ca}$ channels.

**Discussion**

This study assesses the influence of membrane lipid physical properties on the activity and EtOH sensitivity of reconstituted $hso$ channels. The modification of surface charge density, headgroup structure and composition, and acyl chain saturation were addressed in regard basal $hso$ function and EtOH sensitivity. The results presented here provide further support for the notion that the local lipid environment of a channel can modulate its activity and pharmacological properties.
Cholesterol modulation of BK$_{Ca}$ channels does not require negative surface charge or PS headgroup structure.

The ability of cholesterol (CHS) to modulate hslo channel $P_o$ is independent of membrane negative surface charge and PS headgroup structure. Under the conditions tested (+30 mV, 50 μM [Ca$^{++}$]$_{Free}$), CHS reduction of hslo activity approached 80% (Figure 15b). This indicates that sterol modulation of hslo $P_o$ was at least as robust in the POPE/POPC (3:1) background as in the charged POPE/POPS (3:1) background (45% reduction for the same mol% CHS, 0 mV, 50 μM [Ca$^{++}$]$_{Free}$). The inability of CHS to influence channel behavior in pure POPE bilayers is likely to reflect some aspect of the physical properties of this bilayer type, rather than the loss of POPS and its associated charge. For instance, reports aimed at understanding the thermotropic phase behavior of PE/CHS bilayers suggest that the strong electrostatic and hydrogen-bonding headgroup interactions of PE molecules, and PE-CHS hydrophobic mismatch, favor PE/PE binding as opposed to PE/CHS interactions (McMullen et al., 1999). This can cause a limited lateral miscibility, and the appearance of CHS-rich and CHS-poor domains to form in these bilayers (McMullen et al., 1999). This is in contrast to PC-CHS mixtures in which the sterol is miscible and can promote the formation of the liquid-ordered phase (McMullen et al., 1993). It is possible that a limited lateral miscibility of CHS in the POPE bilayer underlies the reduced sterol modulation of BK$_{Ca}$ activity in POPE/23 mol% CHS bilayers. However, the degree of CHS-PE interaction is susceptible to the temperature, amount of CHS, as well as the length and saturation of acyl chains in the mixture. In general, low temperatures, longer, saturated acyl chains, and higher levels of
CHS promote the formation of CHS-rich and CHS-poor domains (McMullen et al., 1999). As in PC-CHS mixtures, CHS can promote the formation of the liquid-ordered phase in POPE bilayers (Pare and Lafleur, 1998), suggesting that the sterol is miscible in the POPE/CHS bilayers used in this study.

**Increases in curvature stress may underlie modulation of basal $\text{BK}_{\text{Ca}}$ activity by cholesterol and POPE.**

The molecular cone shape, and resulting nonlamellar nature, of the PE phospholipid may also influence the ability of CHS to modulate $\text{BK}_{\text{Ca}}$ channel function. The cone shape of PE derives from a small polar headgroup volume relative to the larger volume of its hydrophobic moiety (Israelachvili et al., 1980). The packing density of the polar headgroups for a cone shaped molecule like PE are lower than for molecules like PC, whose large polar headgroup area creates a more cylindrical shape. This is illustrated in Figure 22. Upon the addition of nonlamellar lipids, the negative curvature of a monolayer will increase in an effort to reduce packing energies in the headgroup and acyl chain regions. However, the opposing monolayer will exhibit equal and opposite tendencies, yielding a bilayer with an elastic curvature stress (Gruner et al., 1985; Gruner, 1985; Hui and Sen, 1989). Curvature stress can modify the activity of a variety of transmembrane and membrane-associated proteins, including the channels formed by alamethicin peptides (Keller et al., 1993). Interestingly, CHS is thought to increase curvature stress since the cross-sectional size differences of the polar and non-polar moieties follow the same trend as for PE (Demiel et al., 1972; Lundbaek et al., 1996). It is, therefore, possible that the POPE background has a high initial curvature stress that
A) Lamellar Phase

B) Reverse Hexagonal Phase

Figure 22. Schematic of “cone” and “cylindrical” lipids, and the phases they prefer. Figure adapted from Szule JA, Fuller NL, and Rand RP (2002) Biophys. J. 82(2):977-984. “Cylindrical” lipids like PS and PC form flat, stable monolayers, and pack efficiently into the lamellar (bilayer) phase. PE and CHS have a molecular “cone” shape, for which the lowest energy packing conformation cannot be assumed in a flat lamellar structure. Under certain conditions (such as: high temperature, high degree of acyl chain saturation, or dehydration) these lipids form non-bilayer phases such as the reverse hexagonal phase. However, below the transition from bilayer to non-bilayer phase, these lipids impart on the bilayer a curvature stress implicated in the modulation of several classes of membrane protein. This stress arises from the latent energy stored in the bilayer as a result of forcing these “cone” shaped lipids into a lamellar phase.
can mask further increases in this parameter caused by CHS. The addition of either POPC or POPS to the POPE bilayer may attenuate its curvature stress, and uncover CHS actions on channel function. Indeed, both POPC and POPS can attenuate the nonlamellar nature of POPE, as demonstrated by an increase in the temperature at which the resulting mixture will undergo the transition to an inverted hexagonal phase (Epand and Bottega, 1988).

Lipids that promote curvature stress antagonize ethanol actions on BK$_{Ca}$ channels.

Ethanol is able to reliably potentiate the activity of BK$_{Ca}$ channels in uncharged POPE/POPC membranes (6/7 cases), suggesting that both negative surface charge and PS headgroup structure are not essential in the mechanism of drug action. The degree of channel potentiation by 50 mM EtOH appears lower in this mixture, though not statistically different from POPE/POPS (3:1) membranes (Figure 19b). This could reflect a limited ability of PS charge or structure to tune some aspect of drug action. Regardless, the lack of EtOH modulation in POPE membranes is also attributed to an intrinsic property of the POPE bilayer. Ethanol, as demonstrated by NMR, can bind near the lipid-water interfaces of E. coli-derived PE and disorder the entire length of the acyl chains in the physiologically relevant fluid phase (Barry and Gawrisch, 1994). It therefore seems likely that adequate drug partitioning should occur in our POPE membranes, though differences in acyl chain composition from egg PE must be considered. In addition, short chain alcohols, including EtOH, can stabilize the lamellar structure of egg PE (Tilcock and Cullis, 1987), suggesting the reduction in EtOH
sensitivity of *hslo* channels in POPE membranes is not attributed to an EtOH-induced disruption of bilayer structure.

Both CHS and EtOH modulate *hslo* *P*₀ in POPE/POPC membranes, in a manner qualitatively similar to that seen for the charged POPE/POPS (3:1) bilayer. This suggests net negative membrane surface charge and PS headgroup structure are not essential in the mechanism of action of these agents. The presence of the POPE headgroup itself cannot prevent channel modulation by CHS or EtOH, as it is present in all mixtures tested. However, pure POPE membranes do not support significant modulation of *hslo* activity by CHS or EtOH. When either negatively charged POPS or uncharged POPC are added to the POPE bilayer, the actions of both agents are restored. The ability of both POPS and POPC phospholipids to accomplish this suggests that it is not necessarily attributed to their charge or structure, but the ability of both phospholipids to attenuate the nonlamellar tendency of the pure POPE membrane. In fact, it has been previously shown that mixing with either POPC or POPS can attenuate the nonlamellar tendency of POPE, measured as an increase in the bilayer to inverted hexagonal phase transition temperature (Epand and Bottega, 1988).

**Ternary lipid mixtures, per se, do not antagonize ethanol actions on BK<sub>Ca</sub> channels.**

The addition of 20 mol% POPC to the POPE/POPS (3:1) mixture reduces the basal *P*₀ of *hslo* channels in a similar fashion to a comparable amount of CHS (23 mol%) (Figure 20a). POPC carries the same profile of acyl chains as both POPE and POPS, so the order of the hydrocarbon interior is unlikely to differ dramatically. Relative to the cone shape of PE and CHS, the cylindrical phosphatidylcholine (PC) strongly prefers the
lamellar phases (Stubbs and Slater, 1996). POPC, as a result, would not increase curvature stress. As a result, it is not immediately obvious how the addition of POPC influences basal channel function. It is possible that the altered headgroup interactions of a ternary lipid mixture influence the lateral organization of the bilayer. The basal $P_o$ in the POPE/POPS (3:1) + 20 mol% POPC ternary mixture is similar to that seen in the POPE/POPC (3:1) mixture, suggesting perhaps a preferential association of the channel with POPE and POPC.

In contrast, CHS reduces the EtOH sensitivity of $\text{hslo}$ channels when added (>23 mol%) to POPE/POPS (3:1) mixtures (Figure 12), while similar amounts of POPC (20 mol%) cannot do so (Figure 20b). As indicated above, membrane curvature stress is a unifying theme in the reduced efficacy of EtOH in pure POPE and POPE/POPS (3:1) containing high CHS concentrations. The addition of POPC to the POPE/POPS (3:1) mixture would not be expected to influence curvature stress in a manner similar to CHS, and does not significantly reduce $\text{hslo}$ EtOH sensitivity, consistent with this hypothesis.

Acyl chain order does not directly correlate with changes in basal activity or ethanol sensitivity of $\text{BK}_{\text{Ca}}$ channels.

To assess the influence of bilayer order on the activity and EtOH sensitivity of $\text{hslo}$ channels, we employ here a PE/PS (3:1) mixture containing only mono-unsaturated oleic acid side chains. The presence of only unsaturated chains in the hydrocarbon core of the bilayer cause the mixture to be substantially less ordered than the corresponding POPE/POPS (3:1) bilayer. Figure 21a demonstrates that decreasing the order of acyl chains, without modifying headgroup composition and surface potential, reduces the
basal activity of reconstituted hslo channels. There does not appear to be a direct correlation between bilayer order and hslo open probability, since lipid substitutions expected to increase (23 mol% CHS) (Figure 13) or decrease (DOPE/DOPS 3:1) (Figure 21a) acyl chain order both result in a decrease of basal hslo function.

EtOH is known to disorder acyl chains, though the functional significance of this effect is unclear in regard to modulation of ion channel activity (Chin and Goldstein, 1981). If decreases in the acyl chain order of POPE/POPS (3:1) bilayers underlie the activation of BKCa channels by EtOH, reconstitution of the channels in a less ordered bilayer, like DOPE/DOPS (3:1), would be expected to increase channel activity and enhance the actions of EtOH on channel activity. The data obtained in DOPE/DOPS (3:1) mixtures shown here indicates that neither prediction is borne out experimentally. In the DOPE/DOPS (3:1) background only 40% of the reconstituted hslo channels are potentiated by EtOH, compared to 80% in the POPE/POPS (3:1), and the average potentiation of BKCa activity elicited by EtOH in these bilayer types are not statistically different. As in the modulation of basal Po, these results do not indicate a direct correlation between acyl chain order and BKCa EtOH sensitivity. Therefore, while CHS can antagonize EtOH disordering of phospholipid acyl chains (Chin and Goldstein, 1981) it is unlikely to explain sterol antagonism of BKCa potentiation by EtOH (Figure 12).

This view is strengthened by the observation that pure POPE membranes also antagonize EtOH activation of the reconstituted BKCa channels, and EtOH disordering of PE bilayers has been previously demonstrated (Barry and Gawrisch, 1994).
Ethanol partitioning and acyl chain unsaturation.

It has been demonstrated previously that EtOH partitioning into membranes is maximal at temperatures near the main phase of the bilayer, where there is a dynamic coexistence of both gel and liquid crystalline phases (Jorgensen et al., 1993; Trandum et al., 1999). The transition temperature of the DOPE/DOPS (3:1) mixture is well below room temperature at which these experiments were conducted. Therefore, the membrane will not exhibit the coexistence of gel and liquid crystalline phases theorized to promote EtOH partitioning (Jorgensen et al., 1993). While a smaller percentage of hslo channels were activated by 50 mM EtOH in this background, robust potentiation was elicited in several cases and the overall response was not statistically different from POPE/POPS (3:1). This suggests that even if partitioning is decreased, the impact upon channel sensitivity was not apparent.

Summary

POPE/POPS (3:1) bilayers support BK_Ca channel modulation by EtOH (Figure 9, Figure 11) and CHS (Figure 13), while in POPE membranes the efficacy of both is drastically reduced (Figure 15). The efficacy of EtOH and CHS restored in the neutral POPE/POPC (3:1) background (Figure 18, Figure 19), suggesting that a negative membrane surface potential or PS headgroup structure itself are not required for BK_Ca channel modulation by EtOH and CHS. It is, therefore, an intrinsic property of the pure POPE that masks the action of these agents.

The “cone” molecular shape of lipids like POPE and CHS can increase the curvature stress in the bilayer. Both POPE and CHS-containing membranes support low
basal BK$_{Ca}$ channel activity and both antagonize the actions of EtOH on BK$_{Ca}$ channels. Furthermore, CHS inhibition of basal BK$_{Ca}$ activity is reduced in the POPE background, suggestive that, since their effects are not additive, POPE and CHS modulate a common bilayer property (curvature stress) important for the basal function and EtOH sensitivity of BK$_{Ca}$ channels. Figure 23 provides a model to summarize these concepts.
Monolayer curvature is dependent on the shape ("cone" vs. "cylinder") of the lipids in the mixture. "Cone" shaped lipids, with no extrinsic constraints, will exhibit concave monolayer curvatures to minimize packing energies. This tendency is antagonized by the addition of "cylindrical" lipids in the mixture.

**Figure 23.** Model. "Cone"-shaped lipids promote curvature stress, and also antagonize EtOH actions on reconstituted BKCa channels.
Reference List


DISCUSSION

The influence of lipid environment on the basal function and ethanol (EtOH) sensitivity of ion channels is not easily approached in complex natural membranes, but can be assessed in minimal preparations such as the planar lipid bilayer. This system allows experimental control over both the protein and lipid components in the study of channel physiology. This technique was employed to systematically address the influence of membrane lipid composition on both the basal activity and ethanol (EtOH) sensitivity of cloned human (hslo) large conductance Ca$^{++}$-activated K$^+$ (BK$_{Ca}$) channels.

A complex array of lipid species compose the plasma membrane, which distribute non-randomly into domains within and across bilayer leaflets (Devaux and Zachowski, 1994; London, 2002; Welti and Glaser, 1994). For instance, cholesterol (CHS) and sphingomyelin form lipid raft structures, that can be isolated from natural membranes by virtue of their physical properties (Figure 2; London, 2002). These domains are enriched in signaling molecules and channel proteins (Tsui-Pierchala et al., 2002), including BK$_{Ca}$ (Bravo-Zehnder et al, 2000). Membrane properties such as CHS content, headgroup composition, and lipid surface potential can influence BK$_{Ca}$ channel function (Chang et al., 1995b; Chang et al., 1995a; Moczydlowski et al., 1985; Park et al., 2003; Chapter II and III), indicating that lipid domain partitioning could regulate channel activity. Moreover, the lipid composition of the membrane is tightly regulated (Brown and Goldstein, 1999; Thewke et al., 2000), and it adapts in response to chronic EtOH exposure (Swann, 1987; Taraschi et al., 1991; Wood et al., 1990b). EtOH acutely potentiates BK$_{Ca}$ channels in rat neurohypophysial nerve terminals (Dopico et al., 1996;
Dopico et al., 1999), but this sensitivity is diminished following chronic exposure to the drug (Knott et al., 2002). In addition, BK<sub>Ca</sub> channels from the soma of the same neurons are insensitive, even acutely, to EtOH. The mechanistic basis for these observations is unknown, but we determine here the potential contribution of changes in the membrane lipid environment. CHS is a particularly enticing candidate since this sterol is instrumental in regulating membrane properties (London, 2002; Ohvo-Rekila et al., 2002), and both increases in membrane CHS concentration and alterations in the leaflet distribution (Chin et al., 1978; Omodeo-Sale et al., 1995; Wood et al., 1990b) of CHS occur in response to chronic EtOH exposure. Here, we begin to link chronic EtOH-induced alterations in membrane composition, such as increases in CHS, with the loss of BK<sub>Ca</sub> channel sensitivity to EtOH that also occurs following chronic exposure to the drug. The control of the lipid bilayer composition, and resulting physical properties, afforded by the planar bilayer technique allows inquiry into the manner by which lipid composition influences BK<sub>Ca</sub> channel function.

The major findings of this thesis are: 1) CHS antagonizes the basal activity and EtOH sensitivity of reconstituted BK<sub>Ca</sub> channels in POPE/POPS (3:1) bilayers, 2) The influence on channel function of both EtOH and CHS are drastically reduced upon removal of POPS from the lipid bilayer, 3) This impairment in the pure POPE background is not attributed to the loss of membrane surface potential, 4) The loss of EtOH action on reconstituted <i>hslo</i> channels correlates with the nonlamellar nature of the bilayer, and 5) (Appendix I) Ca<sup>++</sup> pre-exposure of the bilayer or the protein may regulate the EtOH sensitivity of <i>hslo</i> channels.
A more detailed mechanistic interpretation of these observations is found separately within the Discussion sections of Chapters II and III of the thesis. Figure 22 and the model shown in Figure 23 of Chapter III summarize the lipid physical properties that correlate with a reduction in the BK_{Ca} channel EtOH response. Here, we discuss some more general implications of the work. First, and most obvious, the lipid composition of the membrane can influence BK_{Ca} channel function. The basal activity of the channel is inhibited, for instance, by increases in CHS and decreases in negatively charged POPS. Moreover, alterations in lipid physical properties such as acyl chain unsaturation, without changing the headgroup composition of the membrane, influence basal P_{o}. This is evidenced by differences in basal activity between POPE/POPS (3:1) and DOPE/DOPS (3:1) bilayers (Figure 21). This suggests that the physical properties imparted on the bilayer by a particular lipid species can influence the function of transmembrane ion channels. However, since lipids are not modular in structure, the isolation of a single lipid physical property is difficult even in a reductionist planar bilayer system. Regardless, general conclusions can be drawn. Relative to POPE/POPS (3:1) bilayers, the EtOH sensitivity of reconstituted hslo channels is significantly reduced in both pure POPE and POPE/POPS bilayers containing large amounts of CHS (>23 mol%). Both POPE and CHS have a molecular shape conducive, energetically, to the transition from bilayer to nonlamellar inverted phases. Below the temperature for this transition, however, these nonlamellar lipids impart a curvature stress within the bilayer that modulates protein function. This interpretation is most compelling, since differences
in surface charge cannot explain this phenomenon, and the loss of EtOH sensitivity does not correlate linearly with bilayer order (see Chapter III).

Applying these general observations back to the native membrane, we might predict that BK\textsubscript{Ca} channels partitioned into CHS-rich domains would differ in basal activity and EtOH sensitivity from those in the bulk lipid. Such domains seem to exist in natural membranes, and are referred to as lipid rafts (Brown and London, 1998; London, 2002). Moreover, hslo channels transfected into Madin-Darby canine kidney (MDCK) cells can partition into lipid rafts, and this partitioning appears to regulate their sorting to the apical membrane of these polarized cells (Bravo-Zehnder et al., 2000). Raft domains are also emerging as a mechanism to organize and regulate important signaling molecules (Tsui-Pierchal et al., 2002). The experiments described here suggest, in addition, that the physical properties of these CHS-rich domains could regulate the basal activity and EtOH sensitivity of BK\textsubscript{Ca} channels. It should be noted that most data concerning the nature of rafts stems from their biochemical isolation, and relatively little is known regarding their formation, lifetime, and regulation in living cells. The potential for controlling trafficking, organization, and modulation of ion channels and downstream signaling molecules by virtue of CHS-rich membrane domains, however, is tremendous. This becomes doubly interesting when considered in the context of membrane adaptation.

The modulation of membrane lipid composition may reflect a means to maintain homeostatic membrane properties. This phenomenon is widely seen in the adaptation of bacteria to differing environmental conditions (Cronan, Jr., 2002; Hazel and Williams, 1990), perhaps as a means to maintain constant a physical property such as curvature.
stress (Osterberg et al., 1995). In mammals, processes such as aging and chronic EtOH exposure can precipitate changes in membrane lipid composition, including alterations in CHS (Giusto et al., 2002; Schroeder et al., 1996). The vast body of data examining changes in lipid composition following chronic EtOH exposure has largely been interpreted in the context of compensatory adaptations to the acute actions of EtOH on membrane lipids. Interpretation in this way is unsatisfactory, since it is increasingly clear that these acute actions of the drug do not provide a mechanistic explanation for its actions on the nervous system (see Introduction). It may, perhaps, be fruitful to consider the changes in lipid composition as adaptation to the acute actions of EtOH on membrane function as a whole. This would include not just the maintenance of lipid physical properties, but monitoring and adapting to the readout of membrane function as the drug interacts with relevant targets such as ion channels. Plasticity of this nature is a functional hallmark of a neuron. This view serves to reconcile the lack of a unifying theme in the array of lipid changes described (Gustavsson, 1990; Swann, 1987; Taraschi et al., 1991) since neurons, based on their physiological roles, will undoubtedly differ in basal membrane function and properties, as well as their homeostatic set points. A causative link between tolerance to EtOH action on neurons and changes in their lipid composition will require extensive future work. It is an enticing prospect since it is increasingly clear that the cell uses lipids not simply to create a barrier, but to organize and regulate the proteins vital for its function. We begin here with the demonstration that, at least in a simple bilayer system, the lipid environment of an ion channel regulates both its basal function and response to EtOH.
REFERENCES


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APPENDIX A

PRE-EXPOSURE TO HIGH Ca\(^{++}\) CONCENTRATIONS ENHANCES THE ETHANOL SENSITIVITY OF RECONSTITUTED HSLO CHANNELS

Introduction

The large conductance Ca\(^{++}\)-activated K\(^{+}\) (BK\(_{Ca}\)) channel is a target of both acute and chronic ethanol (EtOH) exposure. EtOH potentiates BK\(_{Ca}\) channels in native membranes (Dopico et al., 1996; Knott et al., 2002), ripped-off patches (Dopico et al., 1996; Dopico et al., 1998; Dopico et al., 1999) and planar lipid bilayers (Chu et al., 1998; Figure 7, Figure 8). The persistence of EtOH modulation through the decreasing complexity of these preparations suggests that the drug does not require diffusible second messengers or a complex lipid environment to activate BK\(_{Ca}\) channels. It is more likely that EtOH interacts with the channel protein itself, or at the lipid/protein interface. BK\(_{Ca}\) channels are gated by membrane voltage and increases in the intracellular free Ca\(^{++}\) concentration ([Ca\(^{++}\)]\(_{\text{free}}\)). Increases in [Ca\(^{++}\)]\(_{\text{free}}\) cause a shift in the half-activation voltage (\(V_{1/2}\)) for the channel toward more hyperpolarized values. As a result, these channels may play a vital role in cellular excitability and Ca\(^{++}\)-dependent processes such as vesicle release. Indeed, EtOH potentiation of BK\(_{Ca}\) channels in rat neurohypophysial nerve terminals occurs coincidentally with drug inhibition of peptide hormone release by these terminals. The enhancement of BK\(_{Ca}\) current by EtOH would repolarize the cell, thereby reducing Ca\(^{++}\) influx and, consequently, vesicle release. The efficacy of EtOH in modulating both channel function and peptide release drops sharply in terminals isolated from rats chronically exposed to the drug, suggesting a form of tolerance occurs (Knott et
al., 2000; Knott et al., 2002). The seemingly direct nature of the interaction between EtOH and BK\textsubscript{Ca} channels, in combination with its physiological significance, warrants further study into the mechanism of drug action on the channel.

EtOH is a functional partial agonist of the BK\textsubscript{Ca} channel, with Ca\textsuperscript{++} as the full agonist (Dopico et al., 1998). EtOH potentiates channel activity (agonism), but also antagonizes the ability of Ca\textsuperscript{++} to do so. The BK\textsubscript{Ca} channel response to EtOH has not been tested under zero Ca\textsuperscript{++} conditions. No such interaction with membrane voltage was discernable (Dopico et al., 1998). The mechanistic basis for this observation is unclear. Regardless, it suggests that the [Ca\textsuperscript{++}]\textsubscript{Free} levels at the intracellular face of the channel during the application of EtOH will influence the degree to which the channel responds to the drug.

Here, we address a different aspect of the interplay between Ca\textsuperscript{++} and EtOH, the Ca\textsuperscript{++} "history" prior to EtOH application. In planar lipid bilayer reconstitution experiments, cloned human BK\textsubscript{Ca} (hslo) channels respond robustly to EtOH if incorporated into the bilayer in the presence of \(~50\) \(\mu\text{M}\) [Ca\textsuperscript{++}]\textsubscript{Free}. Following channel insertion the [Ca\textsuperscript{++}]\textsubscript{Free} is reduced to \(~10\) \(\mu\text{M}\) with a chelator, to generate a basal activity from which potentiation by 50 mM EtOH is assessed. Under these conditions the channel activity is routinely increased on the order of five-fold, as shown in Chapter II (Figure 11). Initial experiments demonstrate, in contrast, that hslo channels incorporated into the bilayer and tested directly at \(~10\) \(\mu\text{M}\) [Ca\textsuperscript{++}]\textsubscript{Free}, without prior exposure to 50 \(\mu\text{M}\) [Ca\textsuperscript{++}]\textsubscript{Free}, are not as responsive to EtOH application. Essentially, hslo channels in both experiments are tested for EtOH sensitivity with the same level of Ca\textsuperscript{++} in the bath.
μM [Ca\(^{++}\)]\(_{\text{free}}\)) but only channels pre-exposed to 50 μM[Ca\(^{++}\)]\(_{\text{free}}\) respond significantly to the drug. This suggests prior exposure to higher [Ca\(^{++}\)]\(_{\text{free}}\) may prime or enhance the response of hslo channels to EtOH. We set out to quantify differences in the EtOH response as a function of pre-exposure to high [Ca\(^{++}\)]\(_{\text{free}}\) levels.

**Materials and Methods**

*HEK 293 membrane preparation.* HEK-293 membrane fragments were isolated using a protocol for COS cells (Sun et al., 1994), modified slightly. Briefly, HEK 293 cells stably transfected with hslo cDNA (a gift from Dr. P. Ahring, NeuroSearch A/S, Denmark) were grown to confluence, pelleted, and resuspended on ice in 10 ml of buffer (mM): 30 KCl, 2 MgCl\(_2\), 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 5 ethylene glycol-bis(β-aminoethyl ether)-N,N’,N’,N’-tetraacetic acid (EGTA); pH 7.2. The cell suspension was forced through a 27-gauge needle 4 times, and sonicated at 30% maximum power for 30 sec, twice. The suspension was layered on a 20%-38% sucrose density gradient (in 20 mM MOPS, pH 7.1) and centrifuged at 25,000 rpm for 60 min at 4° C. The band at the 20%-38% interface was collected with a syringe, diluted with bidistilled H\(_2\)O, and centrifuged in a 50.2 Ti rotor at 45,000 rpm for 60 min at 4° C. The resulting pellet was resuspended in 200 μl of buffer (mM): 250 sucrose, 10 HEPES; pH 7.3. Aliquots were stored at -80° C.

*Electrophysiology.* Channels were incorporated by dropping 0.5 μl of the membrane preparation onto preformed bilayers cast from mixtures of POPE and POPS (3:1 w/w). Lipids were dried under N\(_2\) gas, and resuspended in decane, for a final lipid concentration of 25 mg/ml. Bilayers were formed by painting the lipid mixture across a 100 μM hole...
formed in a plastic coverslip (Wonderlin et al., 1990). Capacitance was monitored by the capacitive 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 their Ca\(^{2+}\)-sensor facing the cis chamber were studied. Solutions consisted of (mM): cis, 300 KCl, 10 HEPES, 1.05 CaCl\(_2\), and either 1.45 N-(2-hydroxyethyl) ethylene-diaminetriacetic acid (HEDTA) ([Ca\(^{2+}\)]\(_{\text{free}}\) 15 \(\mu\)M), 1.25 HEDTA ([Ca\(^{2+}\)]\(_{\text{free}}\) 25 \(\mu\)M), or 1.10 HEDTA ([Ca\(^{2+}\)]\(_{\text{free}}\) 50 \(\mu\)M), pH 7.2, and trans, 30 KCl, 10 HEPES, 0.1 HEDTA, pH 7.2. EtOH sensitivity was tested at 10 \(\mu\)M, 15 \(\mu\)M, and 25 \(\mu\)M [Ca\(^{2+}\)]\(_{\text{free}}\). EtOH modification of slo activity is independent of voltage within the range studied here (Dopico et al., 1996). [Ca\(^{2+}\)]\(_{\text{free}}\) in the cis chamber was adjusted using aliquots from either a 300 mM stock solution of CaCl\(_2\), or from a 1M stock of HEDTA. [Ca\(^{2+}\)]\(_{\text{free}}\) values given are nominal, calculated using the Max Chelator Sliders program (C. Patton, Stanford University).

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 either 3 (for dwell time analysis) or 1 kHz (for display and NP\(_0\) determination) 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 6.0.2 (Axon Instr., 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
(Po). N was monitored pre- and post-EtOH by stepping to positive potentials to maximize Po. Experiments showing an increase in N after EtOH addition were discarded. NP o was determined from periods of at least 20 sec of continuous recording. Data are shown as mean±S.E.M. The significance of the difference between means was determined by Student’s t tests.

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 and POPS from Avanti Polar Lipids (Alabaster, AL).

Results

Hslo channels incorporated into 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE)/ 1-palmitoyl-2-oleoyl-phosphatidylserine (POPS) (3:1) bilayers respond robustly to 50 mM EtOH when incorporated into the bilayer in the presence of ~50 μM [Ca++]Free. In these experiments the [Ca++]Free in the bath is then reduced, using N-(2-hydroxyethyl) ethylene-diaminetriacetic acid (HEDTA), to ~10 μM before assessing EtOH sensitivity. This protocol yields, on average, a 5.04 ± 1.35 fold potentiation of channel activity by 50 mM EtOH (Figure 9, Figure 11). Figure 24 demonstrates, however, that hslo channels incorporated and tested directly at lower (10 – 15 μM) Ca++ levels, without prior exposure to 50 μM [Ca++]Free, are not responsive when exposed acutely to 50 mM EtOH. The ratio of NP o in EtOH to control NP o values (NP o EtOH/NP o Control) under these conditions is 0.92 ± 0.09. Incorporation in higher [Ca++]Free, therefore, may promote the sensitivity of hslo channels to acute EtOH exposure. The control NP o values obtained at
Figure 24. Hslo channels incorporated in low (10 – 15 μM) [Ca\(^{++}\)]\(_{\text{free}}\) conditions are less responsive to 50 mM EtOH. Representative traces of a single hslo channel in a POPE/POPS (3:1) bilayer, recorded before and after the addition of 50 mM EtOH. Records were obtained at 0 mV, the channel was incorporated, and tested for EtOH sensitivity, with ~15 μM [Ca\(^{++}\)]\(_{\text{free}}\) on the intracellular side of the bilayer. Data was low-pass filtered at 1 kHz, and sampled at 10 kHz. Arrows denote the closed state.
10 – 15 μM [Ca\textsuperscript{++}]\textsubscript{Free} are similar whether the channels were exposed to 50 μM [Ca\textsuperscript{++}]\textsubscript{Free} in the bath during incorporation, or incorporated directly in lower [Ca\textsuperscript{++}]\textsubscript{Free} (50 μM, chelated to 10 μM [Ca\textsuperscript{++}]\textsubscript{Free}: control NP\textsubscript{o} values = 0.003-0.041, -10 - +50 mV; direct incorporation into 10-15 μM [Ca\textsuperscript{++}]\textsubscript{Free}; control NP\textsubscript{o} values = 0.014-0.043, 0 mV). This suggests no obvious differences in steady state open probability occur as a result of pre-exposure to high [Ca\textsuperscript{++}]\textsubscript{Free}, though a more detailed look at kinetic characteristics is warranted.

We next tested whether channels reconstituted and tested for EtOH sensitivity at an intermediate [Ca\textsuperscript{++}]\textsubscript{Free} value, 25 μM, respond to 50 mM EtOH. This experiment provides two important pieces of information. It allows a rough determination of the range of [Ca\textsuperscript{++}]\textsubscript{Free} pre-exposure that promotes EtOH sensitivity, and it rules out the necessity of the Ca\textsuperscript{++} chelation process itself in promoting \textit{hslo} channel sensitivity to the drug. When incorporated and directly tested for EtOH sensitivity in the presence of 25 μM [Ca\textsuperscript{++}]\textsubscript{Free} (Figure 25a) channels in 9/14 bilayers are potentiated by 50 mM EtOH, yielding an average of 1.74 ± 0.45 fold of control NP\textsubscript{o} values. Exposure to 25 μM [Ca\textsuperscript{++}]\textsubscript{Free} is, therefore, sufficient to prime \textit{hslo} EtOH potentiation. Furthermore, EtOH potentiation is inhibited by increases in bilayer cholesterol (CHS) (Figure 25b), suggesting the mechanism of EtOH action on \textit{hslo} channels reconstituted and tested in this fashion is fundamentally similar to channels that are incorporated at higher [Ca\textsuperscript{++}]\textsubscript{Free} concentrations (Figure 11).
Figure 25. *Hslo* channels reconstituted into POPE/POPS (3:1) bilayers and tested for EtOH sensitivity at 25 μM [Ca$^{++}$]$_{\text{Free}}$ exhibit a response to 50 mM EtOH, and this response is sensitive to membrane CHS levels. Representative traces of *hslo* channel activity in a POPE/POPS (3:1) bilayer (A) and a POPE/POPS (3:1) bilayer containing 23 mol% CHS (B), before and after addition of 50 mM EtOH. Data were obtained at 0 mV (A) and +20 mV (B), with 25 μM [Ca$^{++}$]$_{\text{Free}}$ in the bath. Data was low-pass filtered at 1 kHz, and sampled at 10 kHz. Arrows denote the closed state. C) Scatter plot summarizing data from (A) and (B). Each point represents an experiment from a different bilayer, the Mean(S.E.M.) is indicated above each column.
Figure 26. Brief exposure to 50 μM [Ca$^{++}$]$_{free}$ following incorporation in 15 μM [Ca$^{++}$]$_{free}$ can restore EtOH sensitivity of reconstituted hslo channels. Traces show the same single hslo channel in a POPE/POPS (3:1) bilayer following incorporation at 15 μM [Ca$^{++}$]$_{free}$, during and after exposure to 50 μM [Ca$^{++}$]$_{free}$, and during exposure to 50 mM EtOH. Records were obtained at 0 mV, at the indicated [Ca$^{++}$]$_{free}$ levels. Data are low-pass filtered at 1 kHz, and digitized at 10 kHz. Arrows denote the closed state.
The response to EtOH when channels are incorporated and tested at 25 μM [Ca^{++}]_{Free} is smaller than that seen when channels are incorporated at 50 μM [Ca^{++}]_{Free} and tested at 10 μM [Ca^{++}]_{Free} (1.74 ± 0.45 versus 5.04 ± 1.35, respectively). Two factors may contribute to the smaller NP_{EtOH/Control} seen at 25 μM [Ca^{++}]_{Free} versus 10 μM [Ca^{++}]_{Free}. The first, and most likely, is the prior observation that the EtOH response of BK_{Ca} channel is diminished as the Ca^{++} concentrations in the bath (during exposure to EtOH) increase (Dopico et al., 1998). The second involves the larger control hslo NP_{o} values at 25 μM [Ca^{++}]_{Free} (NP_{o} = 0.008 - 0.314; -25 μM [Ca^{++}]_{Free}, -20 - 0 mV), relative to those obtained at 10 μM (see above). These higher control values could bias the NP_{o} EtOH/NP_{o} Control ratio toward lower values. This is less likely, since the degree of EtOH potentiation is unaffected by the increases in control NP_{o} values caused by membrane voltage (Dopico et al., 1998).

We have shown that incorporation of channels must occur with at least 25 μM [Ca^{++}]_{Free} in the bath in order for EtOH to significantly activate reconstituted hslo channels. We next determine if hslo channels reconstituted in 15 μM [Ca^{++}]_{Free} can be primed with CaCl_{2} to respond to 50 mM EtOH. To do so, channels are incorporated, given a brief pulse of CaCl_{2} (to achieve ~50 μM [Ca^{++}]_{Free} final), and rinsed back to 15 μM [Ca^{++}]_{Free} prior to the addition of EtOH. Figure 26 demonstrates that this treatment is sufficient to restore the hslo channel response to EtOH. This indicates that pre-exposure to high Ca^{++} does not need to occur during channel incorporation, but only at some point before EtOH exposure.
Discussion

Here, we demonstrate that prior exposure to at least 25 μM [Ca\textsuperscript{2+}]\textsubscript{Free} is necessary for hslo channels reconstituted into POPE/POPS (3:1) bilayers to respond significantly to 50 mM EtOH. EtOH is known to serve as a functional partial agonist, and Ca\textsuperscript{2+} a full agonist, of BK\textsubscript{Ca} channels. Increases in [Ca\textsuperscript{2+}]\textsubscript{Free} in the bath solution reduce the potentiation of hslo channels by EtOH (Dopico et al., 1998). However, this is the first demonstration that Ca\textsuperscript{2+} history can influence EtOH sensitivity at a given [Ca\textsuperscript{2+}]\textsubscript{Free} level. Future experiments must determine if the depolarization of membrane voltage can substitute for a pulse of high Ca\textsuperscript{2+}, since it is unclear whether a high channel P\textsubscript{o} or Ca\textsuperscript{2+} itself primes the EtOH response. It is also currently unclear whether the target of the high Ca\textsuperscript{2+} pre-exposure is the channel itself or the lipid bilayer. In addition to increasing channel activity and binding the C-terminal tail of the BK\textsubscript{Ca} channel (Bian et al., 2001), Ca\textsuperscript{2+} interacts with negatively charged phosphatidylserine (PS) headgroups in the bilayer. Ca\textsuperscript{2+} can adsorb to PS-containing membranes (McLaughlin et al., 1981) and causes phase separation when present in millimolar concentrations (Papahadjopulos and Poste, 1975; Tilcock et al., 1988). Therefore, it is important to determine whether this general phenomenon occurs in uncharged POPE/1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) (3:1) bilayers that are not influenced by Ca\textsuperscript{2+} in this manner.
Reference List


