Distinct Sensitivity of Slo1 Channel Proteins to Ethanol

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Received July 13, 2012; accepted October 23, 2012

ABSTRACT

Ethanol levels reached in circulation during moderate-to-heavy alcohol intoxication (50–100 mM) modify Ca^{2+} - and voltage-gated K^+ (BK) channel steady-state activity, eventually altering both physiology and behavior. Ethanol action on BK steady-state activity solely requires the channel-forming subunit slo1 within a bare lipid environment. To identify the protein regions that confer ethanol sensitivity to slo1, we tested the ethanol sensitivity of heterologously expressed slo1 and structurally related channels. Ethanol (50 mM) increased the steady-state activities of mslo1 and Ca^{2+} -gated MthK, the latter after channel reconstitution into phospholipid bilayers. In contrast, 50–100 mM ethanol failed to alter the steady-state activities of Na^+/Cl^- -gated rsls2, H^+ -gated mslo3, and an mslo1/3 chimera engineered by joining the mslo1 region encompassing the N

terminus to S6 with the mslo3 cytosolic tail domain (CTD). Collectively, data indicate that the slo family canonical design, which combines a transmembrane 6 (TM6) voltage-gated K^+ channel (K_v) core with CTDs that empower the channel with ion-sensing, does not necessarily render ethanol sensitivity. In addition, the region encompassing the N terminus to the S0–S1 cytosolic loop (missing in MthK) is not necessary for ethanol action. Moreover, incorporation of both this region and an ion-sensing CTD to TM6 K_v cores (a design common to mslo1, mslo3, and the mslo1/mslo3 chimera) is not sufficient for ethanol sensitivity. Rather, a CTD containing Ca^{2+} -sensing regulator of conductance for K^+ domains seems to be critical to bestow K_v structures, whether of TM2 (MthK) or TM6 (slo1), with sensitivity to intoxicating ethanol levels.

Introduction

Large conductance, Ca^{2+} - and voltage-gated K^+ (BK) channels are ubiquitously expressed in both excitable and nonexcitable tissues, controlling a wide variety of physiologic processes (reviewed in Dopico et al., 2012). Studies on recombinant BK proteins expressed in heterologous systems and native channels in their natural cell environment have both consistently documented that the channel steady-state activity or NPo, i.e., the product of the number of ion channels in the membrane (N) and the channel open probability (Po), is drastically modulated by acute exposure to 50 mM ethanol (Brodie et al., 2007; Martin et al., 2008; Wynne et al., 2009). This concentration is reached in blood during moderate-to-heavy alcohol intoxication (Diamond, 1992). Moreover, data from both recombinant and native BK channels (reviewed in Brodie et al., 2007) demonstrate that ethanol modulation of BK

NPo is concentration dependent, with an EC_{50} of ~ 20 mM—that is, close to the blood alcohol level that constitutes legal intoxication in the US (17.4 mM)—and $E_{\text{max}} \approx 100$ mM, a concentration corresponding to a blood alcohol level that may be fatal in ethanol-naïve mammals (Diamond, 1992). Remarkably, the modulation of BK activity by brief exposure to ethanol concentrations < 100 mM has been reported to contribute to a wide variety of alcohol actions in mammals, including inhibition of vasopressin and oxytocin release from neurohypophyseal nerve endings, antinociception via drug action on primary sensory dorsal root ganglia neurons, decreased excitability of nucleus accumbens neurons, aortic and cerebral artery constriction, endothelial proliferation, and neuroprotection against ischemic-reperfusion injury (Liu et al., 2004; Brodie et al., 2007; Martin et al., 2008; Wang et al., 2010).

Although significant information has been gathered to document the contribution of ethanol modulation of BK channels to alcohol-induced perturbations of physiology and/or behavior, much less is known about the mechanisms and site(s) of ethanol action on these channels. It has been shown that the final ethanol effect on BK NPo results from the orchestration of several factors, including the phosphorylation of the channel-forming slo1 subunit (Liu et al., 2006), the presence of channel accessory proteins (Martin et al., 2008; Bukiya et al., 2009; Wynne et al., 2009), the cholesterol amount in the membrane where the channel resides (Crowley

This work was supported by the National Institutes of Health National Institute on Alcohol Abuse and Alcoholism [Merit Award R37 AA11560]; and by a postdoctoral fellowship from the Neuroscience Institute at the University of Tennessee Health Science Center.

Data from this work were presented as a poster: Liu J, Bukiya A, Kuntamallappanavar G, Singh A, and Dopico A (2012) Calcium-sensing, regulatory of conductance for potassium (RCK) domains are critical to embed SLO1 and related ion channel proteins with sensitivity to intoxicating ethanol levels. The Annual Meeting of the Society for Neuroscience; 2012 Oct 13–17; New Orleans, LA.

dx.doi.org/10.1124/mol.112.081240.

ABBREVIATIONS: BK, large conductance, calcium- and voltage-gated potassium; CTD, cytosolic tail domain; MthK channel, prokaryotic potassium channel from *Methanobacterium thermoautotrophicum*; G, macroscopic channel conductance; KOH, potassium hydroxide; N, number of ion channels in the membrane; NPo, index of channel steady-state activity; Po, channel open probability; POPE, 1-palmitoyl-2-oleoyl phosphatidylethanolamine; POPG, phosphatidylglycerol; RCK, regulator of conductance for potassium; TM, transmembrane.

et al., 2003; Bukiya et al., 2011; Yuan et al., 2011a), and the type of phospholipid species in the lipid membrane (Crowley et al., 2005; Yuan et al., 2011a). However, work from reductionist systems has demonstrated that ethanol action on BK channels requires a minimum set of targets: the slo1 subunit and a one- or two-species phospholipid bilayer (Crowley et al., 2005; Brodie et al., 2007; Yuan et al., 2011a). In spite of these advances, the channel protein region

that confers ethanol sensitivity to the slo1 channel remains unidentified.

Slo1 subunits share significant homology with purely voltage-gated K^+ channels of the six transmembrane domain (TM_6) superfamily (K_V channels) (Latorre and Brauchi, 2006; Salkoff et al., 2006; Lee and Cui, 2010) (Fig. 1A). In contrast to slo1, K_V channels are not universally sensitive to ethanol exposure. Moreover, the ethanol sensitivity of K_V channels is

Fig. 1. Msl01 but not rsl02.1:rslo2.2 channels increased activity (NPo) in response to acute exposure to intoxicating ethanol concentrations, indicating that an ion-sensing cytosolic tail is not sufficient to provide high ethanol sensitivity to slo channels. (A) illustration depicting the schematic structure of a msl01 monomer inserted in the membrane, including sequence of msl01 TM segments and functional domains (top), and single-channel recordings from an I/O patch expressing msl01 channels (bottom). Records were obtained before (top trace), during (middle trace), and immediately after (bottom trace) patch exposure to bath solution containing 50 mM ethanol; $V_m = +60$ mV, free $Ca^{2+}_i \approx 0.3$ μ M. (B) illustration depicting the schematic structure of an rsl02 monomer inserted in the membrane, including the sequence of rsl02 TM segments and functional domains (top), and single-channel recordings from an I/O patch expressing rsl02.1:rslo2.2 channels (bottom). Records were obtained before (top trace), during (middle trace), and immediately after (bottom trace) patch exposure to the bath solution containing 50 mM ethanol; $V_m = +50$ mV, $Na^+_i = 80$ mM. In (A and B), channel openings are shown as upward deflections; arrows indicate the baseline. (C) averaged NPo ratios from msl01 ($n = 8$) versus rsl02.1:rslo2.2 ($n = 8$) channels in the presence (NPo_{Ethanol}) and absence (NPo_{Control}) of 50 mM ethanol. A dotted line underscores the lack of ethanol effect level. * $P < 0.05$, different from msl01. Rsl02.1:rslo2.2 channels were also resistant to 100 mM ethanol exposure ($n = 9$) (not shown).

consistently lower than that of slo1 channels, with EC_{50} close to 200 mM (Covarrubias et al., 1995; Finol-Urdaneta et al., 2010). Thus, it seems natural to hypothesize that the higher ethanol sensitivity of slo1 channels is due to the existence of protein structures present in slo1 and absent in other members of the TM6 K^+ channel superfamily.

In addition to the TM6 transmembrane K_V core, slo1 subunits contain a TM segment (S0) leading to an extracellular N terminus and a large cytosolic tail domain (CTD) (Lee and Cui, 2010; Yuan et al., 2011b) (Fig. 1A). The *Slo1* gene, however, is not unique in encoding K^+ channels that include large protein regions additional to the TM6 transmembrane K_V core. Channel-forming proteins similar to slo1 are encoded by the *Slo2.1* (*Slick*), *Slo2.2* (*Slack*), and *Slo3* genes, their protein products rendering a basic phenotype shared with slo1: high conductance for K^+ and dual regulation of gating by transmembrane voltage and ion recognition (Xia et al., 2004; Salkoff et al., 2006). Although the tissue expression and functional role of the slo2 and slo3 channels have been significantly elucidated (Dryer, 1994; Schreiber et al., 1998; Salkoff et al., 2006; Santi et al., 2006; Yang et al., 2007; Lu et al., 2010; Wojtovich et al., 2011), it remains unknown whether the unique ethanol sensitivity of slo1 channels extends to the other members of the Slo family.

In this study, we addressed the aforementioned question by probing slo1, slo2, and slo3 channels in the same expression system using the ethanol concentrations reached in blood during moderate-to-heavy intoxication. Furthermore, we applied ethanol to engineered slo1/slo3 chimeric proteins to narrow down our identification of the ethanol-sensing elements in the slo channel family. Finally, prokaryotic channels that are homologous to eukaryotic channels known to be relevant targets of ethanol have been recently used to obtain structural information on protein channel regions involved in ethanol-sensing (Aryal et al., 2009; Howard et al., 2011). Thus, we took advantage of the structural and gating similarities between slo1 and the Ca^{2+} -gated, TM2 K^+ channel from *Methanobacterium thermoautotrophicum* (MthK channel) and addressed the ethanol sensitivity of the latter.

Present data demonstrate that the ethanol sensitivity of slo1 channels is shared with Mthk but not with slo2 or slo3 channels (Table 1), indicating that 1) the N-terminal S0 region present in slo1 channels is not necessary for ethanol action, and 2) the presence of an ion-sensing CTD is not sufficient either. Rather, a CTD specifically containing Ca^{2+} -sensing regulator of conductance for K^+ (RCK) domains seems sufficient to provide ethanol sensitivity to K_V core structures, whether of 2 or 6 TM domains.

TABLE 1

Key structural features and ethanol sensitivity of slo1 and related ion channel proteins

Sensitivity was defined as a reversible increase in channel steady-state activity (NPo) in response to ethanol exposure.

Channel	S0 Domain	RCKs	Activating Ion (Ligand)	Sensitivity to 50 mM Ethanol
mslo1	Present	Present	Ca^{2+}	Yes
rslo2.1/2.2	Absent	Present	Na^+	No
mslo3	Present	Present	OH^-	No
mslo1/mslo3 chimera	Present	Present	OH^-	No
MthK	Absent	Present	Ca^{2+}	Yes

Materials and Methods

cRNA Preparation and Injection into *Xenopus laevis*

Oocytes. We prepared cDNA coding for mouse brain slo1 (mslo; mbr5) as previously described elsewhere (Liu et al., 2008). Human slo2.1, rat slo2.1 and 2.2, and mouse slo3 cDNAs inserted into pOx vector were generous gifts from Len Kaczmarek (Yale University School of Medicine, New Haven, CT) and Larry Salkoff (Washington University School of Medicine, St. Louis, MO). The slo1/3 chimera was constructed by swapping mslo1 and mslo3 sequences immediately after S6, following methods described elsewhere (Xia et al., 2004). In all cases, the cDNA-containing vectors were linearized with NotI and were transcribed using T3 polymerase and the mMessage mMachine kit (Ambion, Austin, TX). Oocytes were removed from *X. laevis* and were prepared as described elsewhere (Dopico, 2003; Liu et al., 2008). Mslo1, mslo3, mslo1/3, and a mixture of rslo2.1:rslo2.2 (1:1) cRNA were dissolved in diethyl polycarbonate-treated water at 15–50 ng/ μ l; and 1- μ l aliquots were stored at -70°C . Oocytes were injected with cRNA using methods previously described elsewhere (Dopico, 2003; Liu et al., 2003, 2008).

Patch-Clamp Recordings. The interval between cRNA injection of oocytes and ion current recordings was 48 to 72 hours. Oocytes were prepared for patch-clamping, and single-channel or macroscopic currents were recorded from inside-out (I/O) patches as previously described (Dopico, 2003; Liu et al., 2008).

Mslo1. Both bath and electrode solutions contained the following (mM): 135 potassium gluconate, 5 EGTA, 1 $MgCl_2$, 3.84 $CaCl_2$ ($[Ca^{2+}]_{free} = 0.3 \mu M$), 15 HEPES, and 10 glucose at pH 7.35. Nominal $[Ca^{2+}]_{free}$ was calculated with MaxChelator Sliders (C. Patton, Stanford University, Stanford, CA) and was validated experimentally using Ca^{2+} -selective electrodes (Corning Inc., Corning, NY) (Dopico, 2003).

Rslo2.1:Rslo2.2. Both bath and electrode solutions contained the following (mM): 130 potassium gluconate, 5 EGTA, 10 HEPES, and 29 glucose as well as variant amounts of sodium gluconate (10, 70, and 140 mM) at pH 7.3 (Santi et al., 2006).

Mslo3 and Mslo1/3 Chimeras. Macroscopic current mediated by mslo3 was evoked by 0.2-second, 10-mV depolarizing steps from -50 to 250 mV using a leak subtraction protocol P/4. Peak current amplitude was measured at 0.175–0.2 seconds after the start of the pulse, and conductance (G)-V plots were constructed. For each patch, G at any given ionic condition was normalized to G_{max} observed over all conditions (Xia et al., 2004). For both macroscopic current and single-channel recordings, the intracellular solution contained the following (mM): 140 K^+ -methanesulfonate, 20 potassium hydroxide (KOH), and 10 HEPES (H^+), titrated with methanesulfonic acid or KOH to pH 6.0, 7.0, 8.0, or 9.0, corresponding to $[OH^-] = 0.01, 0.1, 1,$ and $10 \mu M$, respectively. The extracellular solution contained the following (mM): 140 K^+ -methanesulfonate, 20 KOH, 10 HEPES (H^+) and 2 $MgCl_2$, titrated with methanesulfonic acid to pH 7.0 ($[OH^-] = 0.1 \mu M$) (Xia et al., 2004).

After excision from the cell, the membrane patch was exposed to a stream of ethanol-free bath solution for several minutes before exposing the patch to 50 mM ethanol for 1 to 2 minutes. This brief exposure ensures accurate determination of ethanol action before the system develops “acute tolerance” to ethanol. Then, the membrane patch perfusion was switched back to the ethanol-free bath solution (washout). Solutions were applied onto the intracellular side of the membrane patches using a pressurized DAD12 system (ALA Scientific Instruments, New York, NY) via a micropipette tip with an internal diameter of 100 μm . Experiments were performed at room temperature (21°C). Currents were acquired with an EPC8 amplifier (HEKA Electronics, Lambrecht/Pfalz, Germany) at 1 kHz. Data were digitized at 5 kHz using Digidata 1320A and pCLAMP 8.0 (Molecular Devices, Sunnyvale, CA).

MthK Protein Preparation and Planar Artificial Bilayer Recordings. MthK channel cDNA was a generous gift from Dr. Brad S. Rothberg (Temple University, Philadelphia, PA). MthK cDNA inserted into the pQE70 vector (Qiagen, Valencia, CA) was transformed

into SG13009 *E. coli* competent cells (Qiagen), which were grown in Luria-Bertani media containing 100 $\mu\text{g/ml}$ ampicillin and 25 $\mu\text{g/ml}$ kanamycin. Protein expression was induced by incubating the cells with 400 μM IPTG (Sigma-Aldrich, St. Louis, MO) for 4 to 5 hours. Protein was purified from the cell extract using an Ni-NTA spin column (Qiagen). MthK protein was then further purified with Superdex-200 (GE Healthcare, Piscataway, NJ) and concentrated by using a 50,000 MWCO Amicon concentrator (Millipore, Billerica, MA). MthK channel protein for the bilayer recording was prepared in 1-palmitoyl-2-oleoyl phosphatidylethanolamine (POPE): phosphatidylglycerol (POPG) 3:1 (wt/wt) liposomes, as described elsewhere (Zadek and Nimigean, 2006), with aliquots being stored at -80°C . For bilayer formation, we used a POPE:POPG 3:1 (wt/wt) lipid mix, which was dried under N_2 gas and resuspended at 10 mg/ml in decane. Vertical 80–120 pF bilayers were formed by painting the lipid mix across a 200- μm diameter hole in a Deldrin cup (Warner Instruments, Hamden, CT). MthK channels were incorporated into bilayers by adding 10–15 μl of liposome preparation into the bilayer *cis* chamber (corresponding to the intracellular compartment) solution. Recording solutions consisted of (mM): 300 KCl, 10 HEPES, 4 CaCl_2 ($[\text{Ca}^{2+}]_{\text{free}} \approx 2 \text{ mM}$), 2 EDTA, pH 7.0 (*cis* chamber); and 30 KCl, 10 HEPES, pH 7.0 (*trans* chamber). The *trans* chamber was held at ground while the *cis* chamber was held at potentials relative to ground. Only channels with their cytosolic domains oriented toward the *cis* chamber were considered for experimentation, which was determined by inward rectification in response to transbilayer voltage (-100 mV versus $+100 \text{ mV}$). Ion currents were acquired at -100 mV with a BC-525D amplifier (Warner Instruments), low-pass filtered at 1 kHz with the four-pole Bessel filter built in the amplifier, and sampled at 5 kHz with Digidata 1322A and pCLAMP 8 (Molecular Devices). Ethanol was added to the *cis* chamber to a final concentration of 50 mM. After ethanol addition, the chamber was covered with parafilm to minimize ethanol evaporation. Ionic current data in presence of ethanol were continuously recorded for 1 minute immediately after adding ethanol to the bilayer chamber.

Chemicals. Unless otherwise stated, chemicals were purchased from Sigma-Aldrich. Ethanol (100% purity) was purchased from American Bioanalytical (Natick, MA). For liposome formation and bilayer recordings, POPE and POPG were purchased from Avanti Polar Lipids (Alabaster, AL).

Data Analysis. Records were analyzed using Clampfit 9.2 software (Molecular Devices). As the index of channel steady-state activity, we used NPo, which was obtained using the built-in option in Clampfit 9.2. In all cases, NPo, which was obtained from 1 minute of continuous recording under each experimental condition: control (last minute of ethanol-free bath solution before ethanol application), ethanol, and washout (last minute of the 3-minute ethanol washout period). Unitary current amplitudes were obtained from all-points amplitude histograms (Dopico, 2003). Further analysis, plotting, and fitting were conducted using Origin 7.0 (Originlab Corp., Northampton, MA) and InStat 3.0 (GraphPad Software, Inc., San Diego, CA). Statistical analysis was conducted using paired Student's *t* test; $P < 0.05$ was considered statistically significant. Data are expressed as mean $\pm$ S.E.M., and *n* indicates the number of patches/bilayers.

Results

Mslol1 channels were studied in I/O patches from *X. laevis* oocytes, a system where 1) these ion channel-forming proteins retain all major characteristics of BK channels, including their ethanol sensitivity (Liu et al., 2003, 2008; Brodie et al., 2007; Bukiya et al., 2009), and 2) the concentration of activating Ca^{2+}_i , which determines the ethanol response of slo1 channels (Liu et al., 2008; Bukiya et al., 2009), can be tightly controlled. Mslol1 channels were probed with ethanol

at physiologic Ca^{2+}_i levels (0.3 μM) and positive voltage ($+60 \text{ mV}$) to evoke a significant number of channel open events, taking advantage of the transmembrane voltage not modifying the ethanol action on slo1 channels, including mslol1 (Dopico, 2003; Liu et al., 2008). Exposure of mslol1 channels to ethanol at concentrations found in blood during moderate-to-heavy alcohol intake (50 mM) consistently (8 of 8 patches) increased the channel steady-state activity (NPo), which reached 2.3 times the pre-ethanol values and returned to these values within a few minutes of patch exposure to ethanol-free bath solution (Fig. 1, A and C). Ethanol action on NPo occurred in the absence of detectable changes in channel unitary current; the bottom panel of Fig. 1A (top and middle traces) shows almost identical unitary current amplitudes (13.6 ± 0.4 versus $13.8 \pm 0.3 \text{ pA}$) in the absence and presence of ethanol, respectively. Confirming our earlier reports (Liu et al., 2003, 2008), patch perfusion with bath solution or bath solution containing urea to iso-osmotically substitute for ethanol failed to increase mslol1 NPo (not shown).

Next, we explored the ethanol response of slo2 channels, which are phylogenetically related to slo1 (Santi et al., 2006). Slo2 are widely expressed in the heart and neurons, participating in adaptation of neuronal firing rate and slow afterhyperpolarizations that follow repetitive firing (Bhattacharjee and Kaczmarek, 2005; Salkoff et al., 2006) and protecting excitable cells against hypoxia (Dryer, 1994). Like their slo1 counterparts, mammalian slo2 channels display high conductance for K^+ . However, they are insensitive to physiologic levels of Ca^{2+}_i while being sensitive to Na^+_i and Cl^-_i (Bhattacharjee and Kaczmarek, 2005; Salkoff et al., 2006). We used a combination of rslo2.1 (encoded by *Slo2.1* or *Slick*) and rslo2.2 (encoded by *Slo2.2* or *Slack*) cRNAs at a 1:1 molar ratio, which results in heteromeric rslo2.1:rslo2.2 Na^+ -gated K^+ channels (Salkoff et al., 2006; Chen et al., 2009; Zhang et al., 2010). In the presence of activating Na^+_i (i.e., 80 mM sodium gluconate in the solution bathing the intracellular side of the channel), we routinely detected single channel events with unitary current amplitudes of $6.1 \pm 0.5 \text{ pA}$ at $+50 \text{ mV}$, as expected from slo2 channels permeating K^+ (Santi et al., 2006). While responsive to Na^+_i (Fig. 2A), rslo2.1:rslo2.2 channels were consistently unresponsive to bath application of 50 mM ethanol ($n = 8$) (Fig. 1B), a result that contrasts with that of mslol1 (Fig. 1C).

Ethanol action on mslol1 activity, however, is a function of activating ligand concentration: ethanol-induced potentiation turns into channel inhibition as Ca^{2+}_i is increased; the “crossover” (i.e., refractoriness to ethanol) in homomeric BK channels occurs at $\sim 20 \mu\text{M}$ Ca^{2+}_i (Liu et al., 2008). Thus, we next explored whether modifications in activating Na^+_i levels could render rslo2.1:rslo2.2 channels sensitive to ethanol. Within a wide ion concentration range (10–140 mM), rslo2 activity increased monotonically with activating Na^+_i ($\text{EC}_{50} \approx 70 \text{ mM}$), confirming previous studies (Santi et al., 2006; Zhang et al., 2010). At all Na^+_i levels (10–140 mM), however, the channel remained consistently resistant to 50 mM ethanol (Fig. 2B). It should be underscored that slo2 refractoriness to 50 mM ethanol is found at an activating-ion concentration range much wider (considering both actual concentration and modification in channel activity) than that at which Ca^{2+}_i determines different slo1 responses to ethanol as follows: activation, refractoriness, and inhibition (Liu et al., 2008; Bukiya et al., 2009). Therefore, modifications in activating ion

Fig. 2. Slo2.1:slo2.2 channel is ethanol insensitive at a wide range of activating Na⁺_i. (A) single-channel recordings of slo2.1:slo2.2 channels from I/O patches at 10 and 140 mM Na⁺_i. Records were obtained before (top trace within each set), during (middle trace), and immediately after (bottom trace) patch exposure to bath solution containing 50 mM ethanol; V_m = +90 mV. Arrows indicate baseline (all channels closed). (B) averaged NPo ratios from slo1 (0.3 μM Ca²⁺_i, n = 8), versus slo2.1:slo2.2 at 10 mM Na⁺_i (n = 3), 70 mM Na⁺_i (n = 4) and 140 mM Na⁺_i (n = 4) in the presence (NPo_{Ethanol}) and absence (NPo_{Control}) of 50 mM ethanol. A dotted line underscores the lack of ethanol effect level. *P < 0.05, different from slo1.

concentration and eventual changes in channel activity fail to render rslo2.1:rslo2.2 channels sensitive to intoxicating concentrations of ethanol. Finally, rslo2 channels were consistently resistant to exposure to 100 mM ethanol (n = 9,

not shown), a concentration that evokes maximal activation of mslo1 and native BK channels (Brodie et al., 2007; Liu et al., 2008). In synthesis, our data demonstrate that rslo2 channels, which participate in synaptic transmission, action potential waveform shaping, and protection against hypoxia (Dryer 1994; Bhattacharjee and Kaczmarek, 2005; Salkoff et al., 2006), are resistant to direct modulation by levels of ethanol reached in circulation during moderate-to-heavy alcohol intoxication.

The lack of rslo2 channel response to 50–100 mM ethanol contrasts with the ethanol response of mslo1 (Fig. 1, A and C) (Brodie et al., 2007; Liu et al., 2008) and other slo1 channels (Crowley et al., 2005; Brodie et al., 2007; Feinberg-Zadek et al., 2008; Bukiya et al., 2009; Yuan et al., 2011a). From an structural point of view, because the slo1 region comprising the N terminus to the S0–S1 cytosolic loop is not present in slo2 (Fig. 1, A versus B, top panels), the unique sensitivity of slo1 to ≤50 mM ethanol may result from: 1) simple combination of the region comprising the N terminus to the S0–S1 cytosolic loop with the TM6 transmembrane K_V structure, 2) simultaneous presence of the region comprising the N terminus to the S0–S1 cytosolic loop and a long CTD, both joined to the TM6 transmembrane K_V core, and/or 3) specific, Ca²⁺_i-sensing structure(s) present in the slo1 CTD but absent in the slo2 CTD. To examine these possibilities and further address the ethanol response of the different members of the slo channel family, we next explored the ethanol sensitivity of slo3.

In contrast to the wide distribution of slo1 and slo2, the H⁺ sensitive (OH[−]-activated) slo3 channels have been found only in mammalian testis (Salkoff et al., 2006). Similar to the slo1 and slo2 channels, however, expression of slo3 proteins results in high conductance K⁺ channels that can be gated by transmembrane voltage. From a structural point of view, slo3 proteins share with slo1 and slo2 not only a TM6 transmembrane K_V core but also a CTD region that includes two RCK structures (Salkoff et al., 2006). In addition, slo3 proteins contain the S0 segment (Salkoff et al., 2006) (Fig. 3A). In the presence of activating alkaline medium (bath solution pH = 8.0, [OH[−]] = 1 μM), we routinely detected single-channel events with unitary current amplitudes of 2.4 ± 0.3 pA at +80 mV, as expected from slo3 channels (Schreiber et al., 1998; Qian et al., 2002). While sensitive to OH[−]_i (Fig. 4A), mslo3 channels were consistently unresponsive to bath application of 50 mM ethanol (n = 5) (Fig. 3, A and C).

As previously done with mslo1 (Liu et al., 2008) and rslo2.1:rslo2.2 (Fig. 2), we also evaluated whether modifications in the activating ion could alter the ethanol response of mslo3 channels. Thus, we constructed G–V plots in the presence of different [OH[−]]_i. As previously reported (Xia et al., 2004), mslo3 G/V plots were leftward shifted as pH increased (i.e., [OH[−]]_i increased), with an EC₅₀ for activating OH[−]_i ≈ 1 μM (pH ≈ 8.0). More important, across a wide activating [OH[−]]_i range (0.01–10 μM; pH 6.0–9.0), mslo3 channels remained resistant to 50 mM ethanol (Fig. 4B). As found for rslo2 (Fig. 2), mslo3 refractoriness to 50 mM ethanol is observed at an activating-ion concentration range much wider than that at which activating Ca²⁺_i determines the slo1 responses to ethanol (Liu et al., 2008; Bukiya et al., 2009). Therefore, modifications in the activating ion concentration and eventual changes in channel activity fail to render mslo3

Fig. 3. Neither mslo3 nor mslo1:mslo3 chimeric channels increased activity (NPo) in response to acute exposure to intoxicating ethanol concentrations, indicating that the N-end and S0–S1 cytosolic loop regions are not sufficient to provide high ethanol sensitivity to slo channels. (A) illustration depicting the schematic structure of an mslo3 monomer inserted in the membrane, including the sequence of mslo3 TM segments and functional domains (top), and single-channel recordings from an I/O patch expressing mslo3 channels (bottom). Records were obtained before (top trace), during (middle trace), and immediately after (bottom trace) patch exposure to the bath solution containing 50 mM ethanol; $V_m = +80$ mV, pH = 8.0. Mslo3 were also resistant to 100 mM ethanol exposure ($n = 8$) (not shown). (B) illustration depicting the schematic structure of an mslo1/3 chimera monomer inserted in the membrane, including sequence of mslo1/3 TM segments and functional domains (top), and single-channel recordings from an I/O patch expressing mslo1/3 chimeric channels (bottom). Records were obtained before (top trace), during (middle trace), and immediately after (bottom trace) patch exposure to the bath solution containing 50 mM ethanol; $V_m = +80$ mV, pH = 8.0. In (A) and (B), channel openings are shown as upward deflections; arrows indicate the baseline. (C) averaged NPo ratios from mslo1 ($n = 8$) versus mslo3 ($n = 5$), mslo1/3 ($n = 8$) channels in the presence (NPo_{Ethanol}) and absence (NPo_{Control}) of 50 mM ethanol. A dotted line underscores the lack of ethanol effect level. * $P < 0.05$, difference from mslo1. Mslo3 channels were also resistant to 100 mM ethanol exposure ($n = 8$) (not shown).

channels sensitive to intoxicating concentrations of ethanol. Finally, mslo3 channels were consistently resistant to 100 mM ethanol application ($n = 8$, not shown). In synthesis, our results demonstrate that mslo3 channels, which may participate in the physiology of fertilization (Schreiber et al., 1998; Salkoff et al., 2006), are not directly modulated by ethanol levels found in circulation during alcohol intoxication.

The lack of alcohol response of mslo3 channels parallels that of rslo2.1:rslo2.2 and contrasts with data from mslo1 and other slo1 channels (present data and previous work reviewed by Brodie et al., 2007). Because the mslo3 structure includes a region comprising the exofacial N terminus to the S0–S1 cytosolic loop, which is highly homologous to that of mslo1, our results indicate that the aforementioned region attached to a TM6 transmembrane K_V–CTD structure is not sufficient to provide ethanol sensitivity to slo channels. Therefore, the combination of the region comprising the N terminus to the

S0–S1 cytosolic loop with the long CTD, and/or the presence of a specific, Ca²⁺_i-sensing structure(s) distinct of the slo1 CTD but absent in rslo2.1:rslo2.2 and mslo3 are required for such ethanol sensitivity.

Next, we engineered a mslo1/3 chimera by joining the mslo3 CTD with the mslo1 N-end–S0–S6 “core” (Fig. 3B, top). As expected from a slo3 C-tail-containing construct (Qian et al., 2002; Xia et al., 2004), the mslo1/3 chimera was OH[−]-sensitive. In all cases ($n = 8$), however, this chimeric channel was refractory to 50 mM ethanol action (Fig. 3, B and C), indicating that the combination of the region comprising the N terminus to the S0–S1 cytosolic loop with an RCK-containing CTD is not sufficient to bestow the TM6 K_V core with sensitivity to intoxicating ethanol concentrations. Instead, CTD structures within or in the vicinity of the RCKs that are present in the Ca²⁺-sensing slo1 but absent in slo2 and slo3 are required.

Fig. 4. Slo3 channel is ethanol insensitive at a wide range of activating OH^- . (A) single-channel recordings of slo3 channels from I/O patches at 0.01 and 10 μM OH^- . Records were obtained before (top trace within each set), during (middle trace), and immediately after (bottom trace) patch exposure to the bath solution containing 50 mM ethanol; $V_m = +60$ mV. Arrows indicate baseline (all channels closed). (B) slo3 averaged macroscopic conductances at 0.01, 0.1, 1.0, and 10.0 μM OH^- (normalized to the maximum conductance at 10 μM OH^-) in the presence (●) and absence (○) of 50 mM ethanol. Each data point represents the average from no fewer than 3 patches.

Testing ethanol sensitivity in the reciprocal, mslo3/1 chimera would have helped us to determine whether a slo1-type of CTD is sufficient to bestow the TM6 Kv core with the ethanol sensitivity characteristic of BK channels. Unfortunately, we failed to obtain reliable, consistent expression of mslo3/1 channels after insertion into pOx vector followed by

cRNA cytoplasmic injection of *X. laevis* oocytes, or insertion into pcDNA3 vector and transfection of mammalian cells (not shown). Thus, to further test the hypothesis that a Ca^{2+} -sensing, RCK-containing CTD provides ethanol sensitivity to K^+ channels, we evaluated ethanol action on prokaryotic MthK channels, which share some relevant structural features and Ca^{2+} -regulation of gating with slo1 channels (Jiang et al., 2002; Qian et al., 2002). MthK channel-forming subunits result from the combination of 1) a S0-less TM core of two segments that resemble S5 and S6 of the TM6 K_v structure, and 2) RCK-containing, short CTD (Fig. 5A).

After reconstitution into POPE:POPG 3:1 (wt/wt) bilayers, MthK unitary current events were identified by their Ca^{2+} -dependent inner rectification, as previously reported elsewhere (Zadek and Nimigean, 2006). In all independent bilayers tested (4 out of 4), the MthK channels were reversibly activated by 50 mM ethanol, their NPo increasing by an average of 2 times the pre-ethanol values (Fig. 5, A and B). As found repeatedly for mslo1 channels (reviewed in Brodie et al., 2007), the ethanol-induced increase in MthK NPo occurred in the absence of noticeable drug-induced changes in the unitary current amplitude (24.2 ± 3.2 versus 24.4 ± 2.5 pA at -100 mV in the absence or presence of 50 mM ethanol, respectively), which is also evident from inspection of the original records shown in Fig. 5A. The fact that both slo1 and MthK are ethanol sensitive indicates that 1) the region comprising the N terminus to the S0–S1 cytosolic loop is not necessary to provide channel sensitivity to intoxicating levels of ethanol, and 2) a Ca^{2+} -sensing, RCK-containing CTD seems to be a distinct structure that provides ethanol sensitivity to both TM2 and TM6 K^+ channel core structures.

Discussion

Among members of the TM6 superfamily of voltage-gated, outwardly-rectifying K^+ channels, BK (slo1) channels display a distinct sensitivity to ethanol concentrations that correspond to alcohol levels found in blood during alcohol intoxication (10–50 mM); this ethanol modulation of slo1 channel activity results in modification of physiology and/or behavior (reviewed in Brodie et al., 2007). Present data, however, unequivocally demonstrate that slo1 channel sensitivity to *direct* ethanol action (i.e., independent of cytosolic signals, extracellular factors, and ethanol metabolism) does not extend to Na^+ -gated slo2 (Fig. 2) or OH^- -gated slo3 channels (Fig. 4), which are other physiologically relevant members of the slo channel family. Indeed, slo2 and slo3 refractoriness to ethanol was observed at concentrations that evoke maximal modulation of slo1 channel activity (100 mM ethanol), even when evaluated in the same expression system, under similar patch-clamp recording conditions, and across a wide range of channel-activating ion concentration (Figs. 2 and 4).

The reversible potentiation of BK channel activity by 50 mM ethanol shown in this study is similar to findings reported with mslo1 channels expressed in *X. laevis* oocytes (Liu et al., 2003; 2008; Brodie et al., 2007) or after transfection of mammalian Cos1 cells (J. Liu and A. Dopico, unpublished results), and with slo1 proteins cloned from human brain (hslo1) expressed in HEK cells (Feinberg-Zadek et al., 2008) or reconstituted into simple (one or two phospholipid species)

Fig. 5. Similar to mslo1, the Ca²⁺-sensing MthK channel is activated by intoxicating concentrations of ethanol. (A) illustration depicting the schematic structure of an MthK monomer inserted in the membrane, including the sequence of MthK TM segments and functional domains (top), and single-channel recordings after MthK incorporation into planar lipid bilayers (bottom). Records were obtained before (top trace), during (middle trace), and immediately after (bottom trace) bilayer exposure to bath solution containing 50 mM ethanol; $V_m = -100$ mV, $Ca^{2+}_i = 2$ mM. Channel openings are shown as downward deflections; arrows indicate the baseline. (B) averaged NPo ratios from mslo1 ($n = 8$), and MthK ($n = 4$) channels in the presence (NPo_{Ethanol}) and absence (NPo_{Control}) of 50 mM ethanol. A dotted line underscores the lack of ethanol effect level. NS, not statistically significant from mslo1 ($P > 0.05$).

bilayers (Crowley et al., 2003, 2005; Yuan et al., 2011a). Such potentiation is also consistent with the 50 mM ethanol-induced decrease in macroscopic current $V_{0.5}$ observed in G/G_{max} -V plots (where G = conductance) from slo1 channels cloned from rat cerebral artery myocytes (cbv1) and expressed in *X. laevis* oocytes (Bukiya et al., 2009). Collectively, these similarities underscore that slo1 channel-forming subunits

surrounded by a bare phospholipid bilayer suffice to sustain the BK channel's alcohol sensitivity.

Considering the structural similarities between slo1 and slo2 channel proteins, the ethanol refractoriness of rslo2.1/rslo2.2 channels is quite remarkable. Like all other members of the superfamily of voltage-gated, outwardly rectifying K⁺ channels, slo1 and slo2 proteins include a transmembrane TM6 core. Additionally, each slo1 or slo2 (whether rlo2.1 or rslo2.2) channel-forming protein contains an ion-sensing CTD with its two RCKs (Fig. 1B) (Jiang et al., 2002; Yusifov et al., 2008; Lee and Cui, 2010; Yuan et al., 2011b), the presence of activating ion being necessary for ethanol modulation of mslo1 NPo (Liu et al., 2008). On the other hand, ethanol probing of chimeric channels made by swapping the mslo1 and bslo1 "core" (in this case, N terminus to S8–S9 linker) and the distal tail region (S9 to C terminus) showed that the former determines the channel's ethanol response (Liu et al., 2003), with CamKII phosphorylation of Thr106 in the bslo S0–S1 loop operating as a modulatory mechanism of alcohol action (Liu et al., 2006). Collectively, these previous findings led us to raise the hypothesis that the response of slo1 channels to intoxicating levels of alcohol is due to the presence of structures in the channel-forming subunit that are additional to the TM6 transmembrane core of purely gated-voltage K⁺ channels, that is, the region that comprises the N terminus to the S0–S1 cytosolic loop and/or the long CTD.

Similar to their slo1 counterparts, slo2 channel-forming subunits contain two RCKs in tandem. Moreover, identification of a Na⁺-coordination site ("sensor") located within the RCK2 domain of rslo2.2 (Zhang et al., 2010) and several Ca²⁺-coordination sites ("sensors") near the RCKs in the slo1 CTD (Yusifov et al., 2008; Lee and Cui, 2010; Yuan et al., 2011b) buttresses the concept that RCK domains in the CTD of slo channels couple ion-sensing to channel gating (Xia et al., 2004; Lee and Cui, 2010; Zhang et al., 2010). Thus, the fact that slo2 channels are unresponsive to 50 mM ethanol indicates that the presence of RCK(s)-containing CTD that empowers the TM6 transmembrane K_V structure with ion-sensing does not necessarily empower this structure with sensitivity to intoxicating levels of ethanol. Moreover, the refractoriness of mslo3 and the mslo1/3 chimera to ethanol (Figs. 3 and 4) indicates that the combination of the region comprising the N terminus to the S0–S1 cytosolic loop with an RCK-containing CTD is not sufficient to bestow the TM6 K_V core with sensitivity to intoxicating ethanol concentrations. Collectively, our present results from the mslo1, rslo1/rslo2, mslo3, and mslo1/3 channels led us to conclude that the slo family canonical design, which combines a TM6 K_V core with CTDs that empower the channel with activating ion-sensing, does not necessarily render sensitivity to intoxicating levels of ethanol.

In contrast to slo2 and slo3, MthK channels showed an ethanol response that is similar to that of mslo1 and other slo1 channels (Fig. 5) (Crowley et al., 2005; Brodie et al., 2007; Feinberg-Zadek et al., 2008; Bukiya et al., 2009; Yuan et al., 2011a). Furthermore, as found for mslo1 channels (Fig. 1) (Brodie et al., 2007) and other BK channels in heterologous expression systems or native membranes (Brodie et al., 2007; Liu et al., 2008; Wynne et al., 2009), the MthK NPo change in response to ethanol was not accompanied by any noticeable change in channel unitary conductance (Fig. 5A). Thus, for

both slo1 and MthK channels, ethanol action is restricted to that of a gating modifier.

Slo1 and MthK channel gating, while not identical, share basic features. MthK proteins form a Ca^{2+} -sensing gating ring that includes eight RCKs per tetrameric channel: the four RCK1s (proximal) are located in the CTD of the channel-forming subunits, and the four RCK2s (distal) are assembled separately as soluble proteins (Fig. 5A) (Jiang et al., 2002). Thus, whether from separate polypeptides (as in MthK) or from the same polypeptide (as in slo1), RCK1 and RCK2 define an octameric Ca^{2+} -gating ring per channel unit. Ca^{2+} -sensing by this ring seems to increase the gating ring diameter (Lee and Cui, 2010; Yuan et al., 2011b), favoring channel opening by “tugging” on the pore domain via the S6-RCK1 linker, which acts like a spring (Niu et al., 2004). Differential ethanol actions on mslo1 and MthK versus rslo2.1/rslo2.2, mslo3, and mslo1/3 channels indicate that a CTD structure(s) within or functionally coupled to RCKs that are involved in Ca^{2+} -sensing and Ca^{2+} -driven gating is required for channel sensitivity to intoxicating concentrations of ethanol. Thus, for being a rather nonselective ligand that modulates protein function at mM levels, ethanol seems to discriminate between different ion-driven coupling (Ca^{2+} versus Na^+ , OH^-) to gating processes to alter ion channel activity.

Point mutagenesis, crystallographic data, and computational dynamics have come together to identify residues in both RCK1 and RCK2, including the “ Ca^{2+} -bowl,” that participate in Ca^{2+} -coordination and/or Ca^{2+} -dependent slo1 channel activation. These residues show high affinity for Ca^{2+} (submicromolar to micromolar), and the change in secondary structure that occurs in the RCK site upon high affinity Ca^{2+} -recognition is not mimicked by Mg^{2+} (Yusifov et al., 2008; Lee and Cui, 2010). On the other hand, functional data from MthK indicate that the proposed Ca^{2+} -gate does not open when exposed to Mg^{2+} (Zadeck and Nimigean, 2006). Notably, patch-clamp electrophysiology has demonstrated that ethanol action on slo1 channels is dependent on physiologic levels of Ca^{2+} but not on those of Mg^{2+} (Liu et al., 2008). Thus, our current data identifying a rather selective Ca^{2+} -sensing, RCK-containing CTD as the critical region that endows slo1 and MthK channels with sensitivity to intoxicating ethanol levels provide a structural explanation for previous findings reporting Ca^{2+} dependence of ethanol action on slo1 channels (Liu et al., 2008).

Collectively, our data represent the first step in the identification of distinct regions that bestow BK channels with an ethanol sensitivity that is significantly higher than that of K_V channels. One of the most striking findings of our study, however, is the differential response of mslo1 and mslo3 channels to ethanol in the same expression system under similar experimental conditions, considering the high amino acid sequence homology of the mslo1 and mslo3 paralogs (Schreiber et al., 1998). Primary alignment of channel constructs that are responsive (mslo1 and MthK) and unresponsive (mslo3) to 50 mM ethanol reveals the presence of 37 residues in the CTD that are conserved between mslo1 and MthK but nonconserved in slo3. These residues are scattered along the long slo1 CTD. Thus, future studies combining computational dynamics, point mutagenesis, and electrophysiology on slo1 constructs where identified residues are substituted with amino acids having variant

side-chain volume and/or chemical properties will be conducted to pinpoint the structural elements that define an ethanol-recognition site(s) in slo1. Based on the importance of Ca^{2+} -sensing (via RCK1 and RCK2) in the ethanol sensitivity of mslo1 channels and, highly likely, MthK channels as well, it is possible to hypothesize that some of the amino acid residues involved in ethanol-sensing are also participants in coupling Ca^{2+} -binding to the channel gate.

Acknowledgments

The authors thank Maria Asuncion-Chin and Bangalore Shivakumar for excellent technical assistance.

Authorship Contributions

Participated in research design: Liu, Bukiya, Dopico.
Conducted experiments: Liu, Bukiya, Kuntamallappanavar.
Contributed new reagents or analytic tools: Singh.
Performed data analysis: Liu, Bukiya, Kuntamallappanavar, Dopico.
Wrote or contributed to the writing of the manuscript: Bukiya, Dopico.

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