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Distinct Regions of the *slo* Subunit Determine Differential BK_{Ca} Channel Responses to Ethanol

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Abstract

Background: Ethanol at clinically relevant concentrations increases BK_{Ca} channel activity in dorsal root ganglia neurons, GH3 cells, and neurohypophysial terminals, leading to decreases in cell excitability and peptide release. In contrast, ethanol inhibits BK_{Ca} channels from aortic myocytes, which likely contributes to alcohol-induced aortic constriction. The mechanisms that determine differential BK_{Ca} channel responses to ethanol are unknown. We hypothesized that nonconserved regions in the BK_{Ca} channel-forming subunit (*slo*) are major contributors to the differential alcohol responses of different BK_{Ca} channel phenotypes.

Methods: We constructed chimeras by interchanging the core and the tail domains of two BK_{Ca} channel-forming subunits (*mslo* and *bslo*) that, after expression, differentially respond to ethanol (activation and inhibition, respectively), and studied ethanol action on these *mbslo* and *bmslo* chimeric channels using single-channel, patch-clamp techniques.

Results and Conclusion: Data from cell-free membranes patches demonstrate that the activity of channels that share a *mslo*-type core-linker (*wt mslo* and the *mbslo* chimera) is consistently and significantly potentiated by acute exposure to ethanol. Thus, a *mslo* tail is not necessary for ethanol potentiation of *slo* channels. In contrast, the activity of channels that share a *bslo*-type core-linker (*wt bslo* and the *bmslo* chimera) display heterogeneous responses to ethanol: inhibition (in the majority of cases), refractoriness, or activation. Overall, our data indicate that the *slo* core-linker is a critical region likely contributing to the differential responses of BK_{Ca} channels to ethanol.

Keywords

Ethanol; Maxi-Potassium Channel; KCNMA1 (*slo*) Gene; Chimeras; Alcohol

Acute exposure to clinically relevant concentrations of ethanol (EtOH) reversibly increases BK_{Ca} channel activity in neurohypophysial terminals, where drug action likely contributes to inhibition of vasopressin release and, thus, diuresis (Dopico et al., 1996). A similar potentiation of BK_{Ca} channels by acute EtOH was reported in GH3 cells (Jakab et al., 1997), and neurons in dorsal root ganglia, where alcohol action on BK_{Ca} channels effectively reduces neuronal excitability (Gruss et al., 2001). On the other hand, intoxicating [EtOH] usually decrease the activity of BK_{Ca} channels from aortic myocytes, which is thought to contribute to alcohol constriction of aortic smooth muscle (Dopico, 2003; Walters et al., 2000). While advance was

made in linking EtOH action on BK_{Ca} channels to alcohol effects in the body, the molecular determinants of differential BK_{Ca} responses to EtOH are unknown.

In supraoptic neurons, EtOH increases nerve ending BK_{Ca} channel activity but fails to modulate cell body BK_{Ca} channels. Data were obtained in cell-free, membrane patches, indicating that the continuous presence of cytosolic messengers is not required for EtOH to evoke differential responses from native BK_{Ca} channels (Dopico et al., 1999).

Native BK_{Ca} channels consist of pore-forming (α , encoded by the *slo1* or KCNMA1 gene), and accessory (β) subunits (Fig. 1A). Following expression in *Xenopus* oocytes, *mslo* (from mouse brain; Butler et al., 1993) channel activity is consistently potentiated by 10–200 mM EtOH (Dopico et al., 1998). In contrast, EtOH action on *bslo* channels (from bovine aortic smooth muscle; Moss et al., 1996) expressed in the same system and under similar conditions, primarily decreases activity. This distinct action of EtOH on *bslo* channels is not modified by the presence of modulatory β_1 -subunits (Dopico, 2003).

Since differential responses to EtOH were found with *slo* expressed in a similar proteolipid environment, we hypothesized that nonconserved regions between *mslo* and *bslo* primarily determine the differential channel responses to EtOH.

The *slo* subunit has seven putative transmembrane segments (S0–6), a long C-terminus, which includes four hydrophobic regions (S7–10), and a “linker” between S8 and S9 (Fig. 1A) (Weiger et al. 2002). This linker connects two functional domains that determine distinct functional properties of *slo* channels (Wei et al., 1994). The “core” domain (S0–8) includes the voltage-sensor (in S4), the pore (between S5 and S6), and the RCK structure, which contributes to divalent sensitivity (Jiang et al., 2001). On the other hand, the “tail” domain (S9–10) includes the “Ca⁺⁺-bowl,” a major determinant of the channel Ca⁺⁺ sensitivity (Schreiber and Salkoff, 1997). Primary sequence alignment of *mslo* (mbr5) and *bslo* shows that nonconserved regions are scattered all around core, linker and tail (Fig. 1A). The *least* conserved region, however, is the C terminus, a modulatory region located in the tail: in *mslo* this region consists of 60 residues; the first 8 being substituted (4 are nonconserved) and the remaining absent in *bslo*, resulting in a shorter C-end (Butler et al., 1993; Moss et al., 1996).

Thus, to identify which region of the subunit is the primary structural determinant of channel responses to EtOH, “*bmslo*” and “*mbslo*” chimeras were constructed by swapping the *mslo* core-linker with the *bslo* tail (Fig. 1B). The chimeras were expressed in oocytes and their basic functional properties as BK_{Ca} channel-forming proteins were determined. Then, we evaluated *mbslo* and *bmslo*, as well as wild-type (*wt*) *mslo* and *bslo*, channel responses to EtOH under identical conditions after expression in the same batches of oocytes.

METHODS

Construction of Chimeras and Channel Expression

DNAs encoding *mslo* mbr5 variant (AAA39746.1), and *bslo* (AAB03663.1) subunits inserted into the pBluescript vector were cut with *Cla*I and *Not*I and reinserted into the pBscMXT vector for oocyte expression. The nucleotide regions coding for the *mslo* and *bslo* core-linker and tail (Fig. 1A) were separated by *Bcl*I and *Not*I, respectively. Then, the tail was interchanged after direct ligation of the *mslo* core-linker with the *bslo* tail (*mbslo*) and vice versa (*bmslo*; Fig. 1B). Wild-type and chimera DNAs were linearized with *Sal*I and *Xba*I, and cRNAs were synthesized in vitro using T3 polymerase (mMESSAGE mMACHINE; Ambion). *Mslo* and *bslo* DNAs were generous gifts from Lawrence Salkoff (Washington University) and Ed Moczydlowski (Yale University).

Xenopus laevis females were maintained in artificial pond water on a 12-hr light/dark cycle. Isolation and injection of *Xenopus* oocytes were performed as described (Dopico et al., 1998; Dopico, 2003). Wild-type and chimeric cRNAs (0.1–0.5 $\mu\text{g}/\mu\text{l}$; volumes = 18.4–23 nl) were injected into oocytes with an automated microinjector (Drummond). Patch-clamp recordings were conducted 2–4 days following cRNA injection.

Single-Channel Recordings Single BK_{Ca} channel currents were recorded in inside-out (I/O) patches using patch-clamp techniques as described (Dopico et al., 1998). The bath solution contained (mM): 130 K^+ gluconate, 2.63 CaCl_2 , 1 MgCl_2 , 5 EGTA, 15 HEPES, pH 7.4; free $[\text{Ca}^{++}] \approx 100$ nM. The electrode solution contained (mM): 130 K^+ gluconate, 5.22 CaCl_2 , 2.28 MgCl_2 , 5 EGTA, 1.6 HEDTA, 15 HEPES, pH 7.4; free $[\text{Ca}^{++}] = 11 \pm 0.6 \mu\text{M}$. The free $[\text{Ca}^{++}]$ was calculated with Max Chelator Sliders (C. Patton, Stanford University) and, for solutions where nominal free $[\text{Ca}^{++}]$ was >300 nM, determined with a pair of Ca^{++} -selective and reference electrodes (Corning). Pipettes were pulled from glass capillaries (Drummond), coated with Sylgard 184 (Dow Corning) to reduce capacitance and noise, and fire-polished with a microforge (WPI, MF200) to give a final tip resistance of 5–8 $\text{M}\Omega$ when filled with electrode solution. A Ag/AgCl electrode was used as ground electrode.

After excision from the oocyte, the membrane patch was exposed to a stream of bath solution containing either EtOH or urea isosmotically replacing alcohol (control) flowing from a “sewer” micropipette (1-mm diameter; WPI). Deionized (100% purity) EtOH (American Bioanalytical) was freshly diluted in bath solution immediately before each experiment. Patch excision and EtOH/urea application (≤ 1 min) were conducted as described elsewhere (Dopico, 2003). All experiments were carried out at 20–25°C.

Unitary currents were measured using an EPC8 amplifier (List), low-pass filtered at 1 kHz with an 8-pole Bessel filter (Frequency Devices 902), and sampled at 10 kHz. Data were acquired and analyzed using pClamp 8 software and a 1320 Digidata interface (Axon). Voltages given correspond to the potential at the intracellular side of the patch.

Data Analysis The product of the number of channels in the patch (N) and the probability that a channel is open (P_o) was used as index of channel steady-state activity. NP_o was calculated from all-points amplitude histograms as described elsewhere (Dopico et al., 1996). Channel mean open time (t_o) was calculated by weighting the time constants obtained from the open times distribution. Dwell time histograms were constructed using the half-amplitude threshold criterion, and an F Table ($p < 0.01$) was used to determine the minimum number of exponential components to appropriately fit dwell-time histogram data (Dopico et al., 1998). NP_o and t_o values were obtained from periods of at least 20 sec of continuous channel activity recording. Data are expressed as mean $\pm$ SEM. Since each oocyte was patched only once, n = number of membrane patches = number of oocytes. Statistical analysis of data was conducted according to Glantz (2001).

RESULTS AND DISCUSSION

Expression of *mbslo* or *bmslo* subunits in *Xenopus* oocytes led to single channel events that displayed all major characteristics of BK_{Ca} unitary currents when recorded in I/O patches. First, the NP_o of both chimeric channels obtained at any given voltage increased similarly with increases in $[\text{Ca}^{++}]_{\text{ic}}$ (Fig. 2). Second, at a fixed free $[\text{Ca}^{++}]_{\text{ic}} (\sim 100 \text{ nM})$, the voltage- NP_o relationship of both chimeric channels could be described by a Boltzmann function, in which at low NP_o , the $\ln(\text{NP}_o)$ vs. voltage plot is linear. Thus, the inverse of the slope is the potential needed to produce an e-fold change in NP_o (Dopico et al., 1996): $13 \pm 2 \text{ mV}$ ($n = 3$) and $14 \pm 3 \text{ mV}$ ($n = 3$) per e-fold change in NP_o for *mbslo* and *bmslo*. These values are not only similar ($p > 0.05$), but also fall within the range previously reported for both *mslo* and *bslo* channels

(Dopico et al., 1998; Dopico, 2003), other *slo* channels (Butler et al. 1993; Crowley et al., 2003; DiChiara and Reinhart, 1995), and native BK_{Ca} channels (Dopico et al., 1996; Toro et al., 1990). This similarity in the voltage-dependence of gating likely reflects the high identity in the voltage-sensing region (in S4) across *slo* subunits.

Third, unitary current-voltage relationships from both chimeric constructs obtained in 130 mM K⁺ at both sides of the membrane were ohmic from -40 to +60 mV (not shown) rendering similar ($p > 0.05$) slope conductances: 253 ± 17 ($n = 10$) and 254 ± 14 ($n = 10$) pS for *mbslo* and *bmslo*. These values are similar to those reported with *wt mslo* (Dopico et al., 1998) and *bslo* (Dopico, 2003) studied in the same system and under similar conditions. The fact that *mslo*, *bslo*, *mbslo* and *bmslo* channels have the same conductance is a functional correlate of the total identity existing in the primary sequence of the pore-lining region across these *slo* subunits (Butler et al., 1993; Moss et al., 1996).

Finally, the mean open time, largely controlled by the *slocore* (Wei et al., 1994), was determined in both chimeras under identical conditions ($V = 20$ mV; free $[Ca^{++}]_{ic} \sim 100$ nM): 1.6 ± 0.3 and 1.4 ± 0.1 msec for *mbslo* and *bmslo* ($n = 3$; $p > 0.05$, unpaired Student's *t* test).

After establishing that expression of *mbslo* and *bmslo* elicited unitary events that fulfilled the basic characteristics of BK_{Ca} channel unitary currents, we evaluated changes in chimeric channel activity evoked by acute exposure to 100 mM EtOH. This [EtOH] represents the E_{max} for both EtOH inhibition of *bslo* (Dopico, 2003) and EtOH activation of *mslo* channels (Dopico et al., 1998). Chimeric channel responses to EtOH were also compared to those from *wt mslo* and *bslo* BK_{Ca} channels expressed in the same batches of oocytes.

Acute exposure of the cytosolic side of I/O patches to 100 mM EtOH reversibly increased *mslo* channel NP_o in 11 out of 11 patches. In contrast, EtOH evoked varied responses in *bslo* activity: decrease in 5 out of 7 patches (72% of cases), increase in 1 (14%) and no change in another (14%) patch (Fig. 4A). Despite high interpatch variability, data from all samples passed the Kolmogorov-Smirnov normality test ($p > 0.1$). Thus, individual means could be statistically tested with parametric methods, which established that the average responses to EtOH from *mslo* versus *bslo* were markedly different ($p < 0.001$, ANOVA and Tukey-Kramer's test) (Fig. 4B). These findings with *wt slo* channels are identical to those obtained using a wide range of [EtOH] (10–200 mM) with *mslo*, in which NP_o was potentiated in 97% of cases (Dopico et al., 1998), and *bslo*, which displayed varied responses to 3–200 mM EtOH (Dopico, 2003). Since differences between *bslo* and *mslo* were obtained in the same batches of oocytes, current data indicate that differential responses to EtOH from *mslo* and *bslo* channels cannot be explained by differences among batches of oocytes. Rather, since the differential responses were evoked in a similar proteolipid environment, they are likely determined by the existence of nonconserved regions between *mslo* and *bslo*. Ethanol responses of chimeric channels, as shown below, support this hypothesis.

As found with *mslo*, EtOH exposure of I/O patches expressing *mbslo* channels resulted in a fast and reversible increase in channel NP_o (Fig. 3). This response was observed in 93% of cases (13 out of 14 patches; Fig. 4A). Thus, for both *mslo* and *mbslo* we found an increase in channel NP_o in response to EtOH in almost every patch/oocyte. In addition, not only the frequency, but also the average EtOH potentiation of NP_o was statistically similar in *mbslo* and *mslo*: 155 ± 15 and $196 \pm 17\%$ of pre-EtOH controls ($p > 0.05$; ANOVA followed by Tukey-Kramer test) (Fig. 4B). Therefore, the long C-terminus existing in *mslo* but missing in *mbslo* is not necessary for EtOH potentiation of *slo* channel activity.

On the other hand, *bmslo* activity in response to EtOH was decreased in 8 (57%), increased in 4 (29%) and basically unchanged in 2 out of 14 (14%) patches (Fig. 4A). This heterogeneous

pattern of responses to EtOH, the predominant being channel inhibition, is similar to that observed in wild-type *bslo*. In fact, there was no statistical difference ($p > 0.05$) in the average changes in NP_o induced by EtOH between *bmslo* and *bslo* (Fig. 4B). Thus, inhibitory responses evoked by EtOH (Fig. 4A) are only obtained with *slo* constructs that share a *bslo*-type core domain-linker region (Fig. 1A). In contrast, potentiation by EtOH is the only response observed in *slo* constructs sharing a *mslo*-type core-linker region. However, since EtOH-induced channel activation could also be evoked in *bmslo* (Fig. 4A), we conclude that, while the *mslo* core-linker is not necessary for EtOH potentiation of *slo* channel activity, the presence of a *mslo* core-linker “locks” (or shifts) the channel population into one state(s) that is activatable by alcohol.

Alignment of the primary sequences of *mbslo* and *bmslo* in the core-linker shows several, scattered, nonconserved residues: one in the loop between S0 and S1, and five more in the S8–9 linker. In addition, the *mslo* linker includes an IYF insert that is absent in *bslo*. Interestingly, a double S in the *bslo* linker constitutes a consensus site for protein kinase C phosphorylation, a process that modulates both basal BK_{Ca} channel activity (Schubert and Nelson, 2001) and alcohol action on native BK_{Ca} channels (Jakab et al., 1997). Different degrees of phosphorylation from cell to cell might contribute to the variant responses to EtOH observed in *slo* channels having a *bslo* core-linker (*bslo* and *bmslo*). An ongoing systematic study using pinpoint mutagenesis and pharmacology will determine the role of each of these nonconserved residues in the *slo* core-linker and, eventually, their posttranslational modification, in EtOH action on BK_{Ca} channel function.

We previously showed that the distinct EtOH response of *bslo* channels, when compared to *mslo*, is unmodified by coexpression of β_1 subunits (Dopico, 2003), and hypothesized that the differential alcohol responses of *mslo* versus *bslo* should be primarily attributed to nonconserved regions in these *slo* subunits. Now, we demonstrate that the primary structural determinant underlying the differential EtOH responses of *mslo* versus *bslo* channels is not their long, nonconserved C-terminus, but the core domain-linker region. Thus, the primary sequence of the *slo* subunit is a critical factor determining BK_{Ca} channel responses to alcohol.

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Fig. 1.

Depiction of the BK_{Ca} channel and construction of chimeric *slo* subunits. (A) BK_{Ca} channel heterodimer with its pore-forming α (*slo*) and modulatory β subunits. The *slo* pore region (P), RCK structure, Ca⁺⁺ bowl, and core and tail domains joined by the linker are indicated. Arrows point to regions with nonconserved residues between *mslo* and *bslo*. The C-terminus of *slo* is much longer in *mslo* than in *bslo*. The dotted line indicates the point at which *mslo* and *bslo* protein domains were swapped to construct chimeric channels; (B) Construction of chimeric *slo* by swapping the C terminal region of *mslo* and *bslo*. Thus, *mbslo* contains the *mslo* sequence from amino acid 1 to 676 (core and linker) and *bslo* from 677 to 1116 (tail), while *bmslo* contains the *bslo* sequence from amino acid 1 to 676 and *mslo* from 677 to 1169.

bmslo

100nM Ca^{++} →
 $\text{NP}_o=0.005$

1 μM Ca^{++} →
 $\text{NP}_o=0.304$

mbslo

100nM Ca^{++} →
 $\text{NP}_o=0.001$

1 μM Ca^{++} →
 $\text{NP}_o=0.063$

1 s
 0.5 pA

Fig. 2.

The steady-state activity of both *bmslo* (top pair) and *mbslo* (bottom pair) channels increases with increases in internal $[\text{Ca}^{++}]$. Representative traces of activity showing increases in channel NP_o when free $[\text{Ca}^{++}]$ at the intracellular side of I/O patches is raised from 100 nM to 1 μM ($V_m = -20$ mV). Each pair of traces was obtained from the same patch. Arrows indicate the baseline; channel openings are shown as downward deflections. For display, records were digitally filtered at 1 kHz. NP_o values were obtained from periods of at least 20 sec of continuous recording.

Fig. 3.

As found with *mslo*, ethanol routinely increases *mbslo* channel NP_o . Representative single channel recordings obtained before and during the application of 100 mM EtOH to the cytosolic surface of the same I/O patch. Records were obtained >10 min after patch excision ($V_m = 40$ mV). Arrows indicate the baseline; channel openings are shown as upward deflections. For display, records were digitally filtered at 1 kHz. NP_o values were obtained from periods of at least 20 sec of continuous recording.

Fig. 4.

Changes in the steady-state activity of wild type and chimeric channels in response to acute ethanol (100 mM). (A) Acute exposure of the cytosolic side of I/O patches to EtOH evoked varied responses in *bslo* and *bmslo*, the majority of channels being inhibited (see text), while routinely potentiating *mslo* and *mbslo* activity after expression in the same batches of oocytes. Ratios of NP_o values obtained in the presence and absence of EtOH (x100) are shown in a scatter graph, where each data point represents an individual patch/oocyte; (B) Average changes in channel NP_o in response to EtOH are shown as mean ± SEM, where n (number of patches/oocytes) is shown in parentheses at the bottom of each bar graph. In the presence of alcohol, NP_o reaches 67 ± 10 (*bslo*), 105 ± 22 (*bmslo*), 155 ± 15 (*mbslo*), and 196 ± 17% (*mslo*) of pre-EtOH values. *Significantly different when compared to *bslo* ($p < 0.05$); #Significantly different when compared to *bslo* ($p < 0.001$); ¶Significantly different when compared to *bmslo* ($p < 0.01$). Multicomparisons were performed with one-way

ANOVA, followed by Tukey-Kramer's test. In both A and B, a dotted line highlights the point at which NP_o is unchanged by EtOH.