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Modulation of BK channels by ethanol

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Abstract

In alcohol-naïve systems, ethanol (<100 mM) exposure of calcium-gated BK channels perturbs physiology and behavior. Brief (several minutes) ethanol exposure usually leads to increased BK current, which results from ethanol interaction with a pocket mapped to the BK channel-forming slo1 protein cytosolic tail domain. The importance of this region in alcohol-induced intoxication has been addressed in *Caenorhabditis elegans* slo1 mutants. However, ethanol-induced BK activation is not universal as refractoriness and inhibition have been reported. The final effect depends on many factors, including intracellular calcium levels, slo1 isoform, BK beta subunit composition, post-translational modification of BK proteins, channel lipid microenvironment and type of ethanol administration. Studies in *Drosophila melanogaster*, *Caenorhabditis elegans* and rodents show that protracted/repeated ethanol administration leads to tolerance to alcohol-induced modification of BK-driven physiology and behavior. Unveiling the mechanisms underlying tolerance is of major importance, as tolerance to alcohol has been proposed as predictor of risk for alcoholism.

Keywords

ethanol; alcohol receptor; slo1 protein; BK beta subunits; ion channel-lipid interactions; ethanol intoxication; alcohol tolerance and dependence; *Caenorhabditis elegans*; *Drosophila melanogaster*; rodent models of alcohol-driven behavior

Introduction: is the BK channel an alcohol receptor?

Ion channel proteins serve fundamental roles in the physiology of excitable tissues, including initiation and propagation of action potentials (AP), generation of postsynaptic potentials, and electro-chemical coupling leading to neurotransmitter and hormone release. It has been widely documented that alcohol (ethyl alcohol, ethanol) alters the physiology of excitable tissues, including central nervous system (CNS) neurons, and behavior. Moreover, ethanol has pharmacological effects common to those of drugs that act primarily by modifying ion channel protein function (e.g., anxiolytics, barbiturates and volatile anesthetics) (reviewed in Fleming, Harris, & Mihic, 2001; Masters, 2009). Thus, it is not surprising that the literature on interactions between ethanol and ion channel proteins is truly massive. With the goal of narrowing down the number of alcohol receptors based on clinical

relevance, Harris *et al.* (2008) advanced several criteria to be fulfilled by an “ethanol receptor”, which now we slightly modify and adapt to *ion channel proteins*: 1) The function of the ion channel protein must be altered by *in vitro* exposure to 150 mM ethanol (Harris, Trudell, & Mihic, 2008). In most states of the Union, blood alcohol levels (BAL) that constitute legal intoxication correspond to 17.4 mM (0.08 g/dL) while 100–150 mM BAL are usually lethal to most alcohol-naïve mammals, including humans (Diamond, 1992; Thombs, Olds, & Snyder, 2003). Ideally, alteration of channel function by ethanol should be observed in recombinant and native ion channel proteins under physiological conditions; 2) Manipulation of specific amino acids in the channel-forming protein should provide evidence of an ethanol-recognition site of discrete dimensions, as well as information on the chemical bonds participating in ethanol-ion channel protein site interaction(s); 3) Manipulation of the channel-forming protein *in vivo* (using sRNAi or epi/genetic manipulations) should alter ethanol modification of physiological and/or behavioral end points controlled by the ion channel protein under examination; 4) ideally, Structural Biology methods (crystallography, NMR, etc.,) should demonstrate the presence of ethanol molecule(s) at the proposed site(s) within the ion channel protein.

Since the first report on selective and concentration-dependent enhancement of ionic currents mediated by large conductance, voltage- and Ca^{2+}_i -gated K^+ (BK) channels in response to a brief exposure (1–2 min) of rat neurohypophyseal nerve endings to 10–100 mM ethanol (Dopico, Lemos, & Treistman, 1996), functional interactions between ethanol and BK channels have been documented in a wide variety of systems (Table I), which fairly fulfills criterion (1) mentioned above. More recent studies using engineered BK channel-forming slo1 proteins (Bukiya *et al.*, 2014) and genetic screening or mutation of the BK channel-coding gene in *Caenorhabditis elegans* (Slo1) (Davis, Scott, Hu, & Pierce-Shimomura, 2014; Davis *et al.*, 2015) have buttressed the idea of an ethanol-recognition site in the BK channel-forming protein (also termed BK α subunit or slo1 channel protein) by satisfying criteria (2) and (3). While criterion (4) has yet to be met, it should be noted that the Molecular Modeling (MM) and Molecular Dynamics (MD) data from Bukiya *et al.* (2014) were based on crystallographic data of the BK channel cytosolic tail domain (CTD) (wherein the ethanol binding site has been mapped) in both “open” (Ca^{2+}_i -bound; P. Yuan, Leonetti, Pico, Hsiung, & MacKinnon, 2010) and “closed” (Ca^{2+}_i -free; Wu, Yang, Ye, & Jiang, 2010) states of the BK channel. The model of ethanol docking onto a site of discrete dimensions in the mslo1 channel (cloned from mouse brain) (Bukiya *et al.*, 2014) explains previously described features of modulation of BK current by ethanol and related n-alkanols: 1) ethanol action’s dependence on Ca^{2+}_i presence (Liu, Bukiya, Kuntamallappanavar, Singh, & Dopico, 2013; Liu, Vaithianathan, Manivannan, Parrill, & Dopico, 2008) (Fig. 1) and 2) the refractoriness of slo1 channels to n-alkanols longer than heptanol (Chu & Treistman, 1997) (Fig. 2). Finally, the coupling of ethanol action to Ca^{2+} -driven gating, is supported by *in vitro* electrophysiology on mslo1 proteins with substitutions in the channel’s Ca^{2+} sensors, single channel gating models (Liu *et al.*, 2008), and modification of ethanol action following slo1 gene mutations in *C. elegans*, using both behavioral and electrophysiological assays as end points (Davis *et al.*, 2015) (see below). The role of specific residues and bonds that participate in ethanol docking onto mslo1 proteins is shown in Fig. 1. Additionally, Thr 381 in *C. elegans* slo1 (equivalent to hslol Thr 352) determines the overall ethanol

sensitivity of BK channel-forming subunits and slo1-driven behavioral responses in *C. elegans* (Davis et al., 2014). A structural role for this conserved residue within the proposed ethanol-docking discrete site is discussed elsewhere (Dopico, Bukiya, & Martin, 2014).

While a direct slo1 protein-ethanol interaction in presence of activating Ca^{2+}_i (Crowley, Treistman, & Dopico, 2003; Liu et al., 2008; 2013) seems sufficient for slo1 channels to fulfill criterion (1) for a functional alcohol receptor, the final ethanol effect on native BK channel-mediated ionic current is highly variable, with potentiation, inhibition and refractoriness all being reported (reviewed in Dopico et al., 2014). This heterogeneous profile is the result of ethanol targeting a wide variety of molecular entities (ethanol receptors additional to slo1 proteins) and mechanisms that may regulate slo1 channel function itself. Thus, we will succinctly present to the reader the many molecular factors that determine the final response of BK current to clinically relevant concentrations of ethanol. Moreover, demonstration of BK channel involvement in alcohol-altered behavior and/or animals models of alcoholism requires some form of “protracted” (longer than a couple of minutes) or repeated exposure of slo1 channel proteins/coding genes to ethanol. As with other drugs of misuse, the response of a biological system to protracted/repeated ethanol exposure *includes* receptors and mechanisms that are perturbed by ethanol when the alcohol is applied to the system for the first time (Dopico & Lovinger, 2009). Thus, in the first section of this chapter, we will summarize and analyze ethanol actions on BK currents provided that ethanol was applied: a) to an alcohol-naïve system; b) for a brief interval (i.e., a few minutes) and c) just once. The second section of our chapter will concentrate on the genetic, epigenetic and posttranslational mechanisms mediating modifications in the BK channel’s ethanol response that are driven by protracted and/or repeated alcohol exposure. Finally, the third section will analyze involvement of BK channels/coding genes in modification of behavior by ethanol.

Responses of native BK channels to clinically relevant ethanol concentrations in alcohol-naïve systems

Ionic current mediated by *native* BK channels renders all possible outcomes (increase, decrease or refractoriness) when these channels are briefly (< 5 min) exposed to clinically relevant ethanol concentrations (10–100 mM) for the first time, as summarized in Table I. It should be underscored that such heterogeneity can be observed even when native channels are studied within a same neuronal type (albeit within different neuronal domains; e.g., somata vs. axon terminals; (Dopico et al., 1996; Dopico, Widmer, Wang, Lemos, & Treistman, 1999; Wynne, Puig, Martin, & Treistman, 2009); or *Nucleus Accumbens* (NAcc) neuronal somata vs. dendrites; (G. Martin et al., 2004) underscoring that ethanol-BK channel results obtained in a presynaptic domain cannot be simply extrapolated to those in a postsynaptic environment.

In a more general context, the consequences of ethanol action on BK channels in a given excitable tissue (e.g., neurons) may completely differ from another. For instance, ethanol potentiation of BK current reduces action potential (AP) firing rate and thus, decreases the excitability of medium spiny neurons (MSN) neurons in rat and mouse (G. Martin et al.,

2004; G. E. Martin et al., 2008). This action has been linked to ethanol-induced perturbation of motor behavior and alcohol preference (reviewed by Bettinger & Davies, 2014; Treistman & Martin, 2009) (see below “*Adaptations in BK channel/coding genes to protracted or repeated ethanol exposure: from molecular mechanisms to modification in ethanol-induced perturbation of behavior*”). A decrease in AP frequency in response to 40 mM ethanol has also been reported in dorsal root ganglia (DRG) neurons that show positive staining for isolectin B4, a marker for nociceptive neurons. Ethanol also shortens AP duration and increases AP mean threshold, these actions being blunted by selective block of BK channels with the peptide iberiotoxin (Ibtx) (Gruss et al., 2001). Thus, authors of this study proposed that ethanol actions leading to reduced firing and excitability of distinct DRG neurons might contribute to ethanol’s analgesic effect in the peripheral nervous system. In contrast to the studies on N. Acc MSN and DRG neurons, ethanol-induced slo1 expression and the drug interaction with BK channels actually *increases* motor neuron excitability within the *D. melanogaster* giant fiber pathway in *D. melanogaster*. This effect is related to a substantial decrease in the neuronal refractory period, which might provide an explanation for the long-standing counter-adaptive theory of “drug tolerance” (Cowmeadow, Krishnan, & Atkinson, 2005; Ghezzi, Pohl, Wang, & Atkinson, 2010). Finally, pharmacological block of presynaptic BK channels prevents ethanol-induced enhancement of GABAergic *inhibitory* neurotransmission in the rat central nucleus of the amygdala (Q. Li, Madison, & Moore, 2014).

Heterogeneity of ethanol responses across different neuronal types and domains can be largely explain by heterogeneity in the subunit composition and distribution of BK channels themselves (see below). However, disruption of physiology by ethanol’s targeting of BK channels must be analyzed having in mind that ethanol is a very simple molecule, a feature that allows this alcohol to “fit” in many putative docking areas and readily cross membranes. Thus, ethanol has the capability to almost simultaneously target many receptors within a single cell domain. In some cases, ethanol’s targeting of more than one ion channel/receptor population results in synergistic modulation of a given cell process by the alcohol. For example, in rat neurohypophysial terminals, ethanol-induced potentiation of BK current in concert with ethanol inhibition of voltage-dependent Ca^{2+} channels (X. Wang, Wang, Lemos, & Treistman, 1994) have been recognized as a central mechanism leading to inhibition of vasopressin and oxytocin release by such nerve terminals (Dopico et al., 1996; Knott, Dopico, Dayanithi, Lemos, & Treistman, 2002). Likewise, ethanol inhibition of smooth muscle BK channels in concert with ethanol inhibition of local Ca^{2+} sparks driven by ryanodine receptors of type 2 (RyR2) synergistically contributes to blunt Spontaneous Transient Outward Currents (STOCs) eventually leading to endothelium-independent cerebral artery constriction (Bukiya, Liu, & Dopico, 2009; P. Liu, Xi, Ahmed, Jaggar, & Dopico, 2004; Ye, Jian, Jaggar, Bukiya, & Dopico, 2014). In other cases, ethanol functional targeting of BK current is antagonized, and can be overridden, by ethanol actions on other cell targets. For example, ethanol-induced BK channel activation in GH3 and GH4/C1 rat pituitary tumor cells would likely lead to inhibition of hormone release (Jakab et al., 2006; Jakab, Weiger, & Hermann, 1997). In spite of BK current potentiation, ethanol actually increases growth hormone secretion in these cells, which has been attributed to ethanol-

induced cell swelling and increase in Ca^{2+}_i (Jakab et al., 2006; Stojilkovic, Zemkova, & Van Goor, 2005; Strbak, 2006).

Heterogeneity in the impact of ethanol-BK channel interactions on cell function is not limited to neurons/neuroendocrine systems. As mentioned above, such interaction has been demonstrated to evoke cerebral artery constriction in both rat and mouse (Bukiya, Liu, et al., 2009; Bukiya, Vaithianathan, Kuntamallappanavar, Asuncion-Chin, & Dopico, 2011; P. Liu et al., 2004). Likewise, this interaction has been hypothesized to contribute to ethanol-induced aortic constriction (Walters, Covarrubias, & Ellingson, 2000). In both cases, ethanol interaction on smooth muscle BK channels results in ionic current inhibition. In contrast, ethanol-induced BK channel activation of either or both neuronal and cerebral microvascular endothelial cells has been proposed as a key mechanism for the neuroprotective effect of ethanol preconditioning against post-ischemic neuronal injury in mice (Q. Wang, Kalogeris, Wang, Jones, & Korthuis, 2010). Targeting of myocyte BK channels by ethanol is not limited to vascular smooth muscle: a recent study shows that BK channel activation plays a critical role in alcohol-induced relaxation of guinea pig urinary bladder smooth muscle (Malysz, Afeli, Provence, & Petkov, 2014).

Collectively, reported functional consequences of an ethanol-BK channel interaction have been largely limited to neurons, neuroendocrine and smooth muscle cells. A single study, however, has documented that 10–50 mM ethanol potentiates BK currents in human endothelial umbilical vein cells, an ethanol action that leads to increased nitric oxide (NO) production and cell proliferation with eventual bolstering of endothelial function (Kuhlmann et al., 2004). The functional consequences of ethanol action on native BK channels in alcohol-naïve systems are also summarized in Table I. The behavioral consequences of the interactions between neuronal BK channels and ethanol have been recently discussed in detail elsewhere (Bettinger & Davies, 2014) and briefly described in a separate section below.

In spite of the heterogeneities in ethanol-BK channel interactions mentioned in the previous paragraphs, some generalizations can be made. Brief exposure of native/recombinant BK channels to intoxicating levels of ethanol in alcohol-naïve systems is: 1) in most cases, rather selective, with this type of ethanol exposure perturbing BK current without altering other voltage-gated K^+ channels (e.g. other K^+ outward rectifiers of the TM6 superfamily) in the same nerve terminal (Dopico et al., 1996), or even the slo1-related, slo2 and slo3 channels after expression in the same system where slo1 channels have been expressed (Liu et al., 2013); 2) unexplained by a simple osmotic challenge to the channel/membrane as a result of exposure to high mM levels of the alcohol (reviewed in Brodie, Scholz, Weiger, & Dopico, 2007). Indeed, both hypotonicity and 30 mM ethanol lead to cell swelling and BK channel activation in GH4/C1 pituitary tumor cells, yet the former and not the latter increases Ca^{2+}_i -dependent Cl^- currents (Jakab et al., 2006). In general, multiple studies favor the idea that ethanol effect on BK channel function is rather specific, and does not simply reflect changes caused by an osmotic challenge (reviewed in Brodie et al., 2007; Mulholland et al., 2009); 3) due to ethanol itself. Data obtained from isolated membrane patches long after patch excision and from channels reconstituted into artificial lipid bilayers, supported by the mutagenesis and MM-MD data mentioned above, advance the notion that ethanol action on

BK channels is due to ethanol molecules rather than ethanol metabolites. It should be noted however, that acetaldehyde, a major metabolite of ethanol, antagonizes ethanol-induced BK channel activation in excised patches from GH3 cells (Handlechner, Hermann, Fuchs, & Weiger, 2013). Considering that BK channels *in vivo* are likely to be exposed to a mixture of ethanol and acetaldehyde, it is conceivable that some of the *in vivo* effects of ethanol on pituitary cells (and on organ systems in general) result from the contribution of acetaldehyde on top of ethanol itself; 4) concentration-dependent, with $EC_{50}/IC_{50}=22\text{--}24\text{ mM}$ and $E_{\max}\sim 100\text{ mM}$ (Dopico, 2003; Dopico, Anantharam, & Treistman, 1998; Dopico et al., 1996); 5) independent of changes in unitary current amplitude (reviewed in Dopico et al., 2014; see also: Davis et al., 2014; Malysz et al., 2014; Marrero, Treistman, & Lemos, 2015) or change in K^+ selectively over other monovalents (Dopico et al., 1998). Given the conservation of ion channel permeation properties in presence of ethanol, potentiation and inhibition of BK current by the alcohol have been attributed to ethanol-induced increase and decrease in BK channel steady-state activity, respectively. In all studies dealing with ethanol-BK channel interactions, channel “steady-state activity” has been routinely determined from the product of the number of functional channels in the membrane patch under study (N) and the individual open channel probability (P_o) (Dopico et al., 1996). The degree of ethanol modification of NP_o in channels of unknown N has been found identical to that in membrane patches containing a single BK channel (i.e., $N=1$) (Dopico et al., 1998). This parallelism and other considerations that rule out any possible increase in N by a short exposure of a cell-free patch or artificial bilayer expressing BK channels to ethanol leads to the conclusion that change in BK current to brief, low concentrations of intoxicating ethanol levels is solely dependent on drug-induced changes in P_o . These changes are consequence of modifications in both open and closed-times distributions (discussed in more detail in Dopico et al., 2014). A gating analysis of ethanol action on BK channels will be summarized below, framed within the ethanol-dependence on activating Ca^{2+}_i to modify BK function. Changes in N in presence of ethanol seem to only occur when the system is continuously exposed for at least 10 min to the alcohol and/or to repeated, brief ethanol exposures, paradigms that are discussed in the second major section of this chapter.

Molecular determinants of changes in BK channel steady-state activity evoked by clinically relevant ethanol concentrations in alcohol-naïve systems

Table I shows that some of the studies demonstrating heterogeneity of BK current response to a first and brief ethanol exposure were conducted in cell-free membrane patches (reviewed in Brodie et al., 2007; Dopico et al., 2014). The experimental conditions of these studies underscore that the different effects of ethanol on native BK channels are largely independent of Ca^{2+} -release/buffering from internal organelles, continuous presence of freely diffusible cytosolic signals, and cell/systemic metabolism of ethanol. Moreover, comparison of ethanol responses between BK channels in a cell-free, native membrane environment, recombinant *slo1* channels in cell-free membrane patches following heterologous expression, and recombinant or native BK channels following channel-forming protein reconstitution into artificial bilayers of controlled lipid composition (reviewed in Brodie et al., 2007; Dopico et al., 2014) indicates that the molecular factors determining the final BK current response to ethanol are inherent to the BK channel protein complex itself (participating channel subunits and their posttranslational modification), the lipid

microenvironment of the BK channel complex, and, fundamentally, dependent on the presence and amount of Ca^{2+}_i nearby the BK channel Ca^{2+} -sensors. These factors have been discussed in detail elsewhere (Dopico et al., 2014) and summarized below.

Activating ions—Overwhelming amount of data show that brief ethanol exposure of homomeric slo1 channels in alcohol naïve systems results in increased channel steady-state activity/macrosopic current provided that Ca^{2+}_i is present at low μM levels (reviewed in Brodie et al., 2007; Dopico et al., 2014; Mulholland et al., 2009). *In absence of activating Ca^{2+}_i* , that is, when the slo1 channel is gated by voltage/ Mg^{2+}_i , slo1 channels become ethanol-resistant even when probed with fully effective activating ethanol concentrations (100 mM) (Liu et al., 2008). Moreover, combination of amino acid substitutions (5D5N) in the mslo1 CTD “ Ca^{2+} -bowl” and in the high affinity Regulator of Conductance for K^+ (RCK) 1 domain (D362A,D367A), which render both high affinity Ca^{2+}_i -recognition sites nonfunctional, results in a channel that is ethanol-resistant (Liu et al., 2008). In contrast, mutations that render the low affinity, divalent (Mg^{2+}_i) site nonfunctional do not have any impact on the ethanol responsiveness of mslo1 channels (Liu et al., 2008). Consistently with the *in vitro* results on Ca^{2+} -bowl and high affinity RCK1 mslo1 mutants, *in vivo* studies on transgenic *C. elegans* show that WT human slo gene insertion into slo1-null worm mutants rescues the egg-laying and locomotion responses to ethanol (which are blunted in the slo1-null worms) whereas insertion in the same genetic background of hslo containing mutations that render both Ca^{2+} -bowl and high affinity RCK1 nonfunctional did not (Davis et al., 2015). It is noteworthy that insertion of equivalent cslo1 mutants on a slo1-null background renders a different outcome (see Davis et al., 2015 for Discussion). *In vitro* data from mslo1 channels also demonstrate that amino acid substitutions that hamper either the Ca^{2+} -bowl or the high affinity RCK1 site do not modify the ethanol sensitivity of BK channels. Thus, as far as Ca^{2+}_i is able to interact with one of its high affinity, physiological recognition sites present in the slo1 protein CTD, BK channel are ethanol-sensitive (Liu et al., 2008). Consistent with the electrophysiological results from mutated mslo1 channels mentioned above and with earlier data demonstrating that ethanol fails to modify the rate-limiting slope of the V-log NPo relationship (a crude reader of the BK channel voltage-dependence; Dopico et al., 1998; Dopico et al., 1996), a 10-state allosteric gating model underscored that ethanol modified only Ca^{2+} -dependent parameters, such as the channel Ca^{2+} dissociation constants for both open (K_O) and closed (K_C) BK channel conformations. In contrast, Ca^{2+} -independent parameters, such the equivalent gating charge associated with the BK closed-to-open equilibrium (Q) and the closed-to-open equilibrium constant in absence of Ca^{2+}_i and transmembrane voltage (L_0 , an index of “intrinsic gating”) remained unchanged (Liu et al., 2008).

Ethanol action on slo1 channels is not only dependent on the presence of Ca^{2+}_i but also modulated by the amount of this divalent sensed by the channel. Ethanol potentiation of Po and macrosopic current progressively diminishes as concentration of Ca^{2+}_i increases from high nM to low μM levels, finally turning to inhibition of slo1 activity when the divalent reaches several tens of μM to close to 1 mM. For homomeric slo1 channels, whether mslo mbr5 variant or cbv1 (slo1 cloned from rat cerebral artery myocytes), the ethanol-induced activation-to-inhibition “cross-over” occurs at $\sim 20 \mu\text{M}$ Ca^{2+}_i (Bukiya et al., 2009; Liu et al.,

2013), this crossover being heavily modulated by BK regulatory β_1 subunits (see below). An empiric single-channel kinetic model revealed that ethanol-induced inhibition of slo1 activity was related to ethanol-induced facilitation of channel dwelling into Ca^{2+}_i -driven low Po modes (Liu et al., 2008), which can be interpreted as ethanol facilitating Ca^{2+}_i -driven BK channel “desensitization” (Dopico & Lovinger, 2009). A very simple scheme summarizing our current understanding of ethanol and the basic processes that gate the slo1 channel is given in Fig. 3. The gating of BK channels, however, is rather complex: while many parameters of voltage-, intrinsic-, and Ca^{2+} -driven gating can be obtained, full understanding of the slo1 gating behavior requires incorporation of allosteric factors that couple the different equilibria between channel closed-open states, inactive-active voltage sensors, and Ca^{2+} bound-unbound Ca^{2+} -sensing sites on slo1 CTDs of the channel-forming tetramer (Cox & Aldrich, 2000; Horrigan & Aldrich, 1999, 2002). Which specific processes are disrupted by ethanol resulting in modification of channel function and BK current is under current investigation in our laboratory. The structural basis of ethanol activation of slo1 channels and its relation to Ca^{2+}_i is provided under the next subheading.

Ethanol is able to perturb Ca^{2+} -driven gating of slo1 and *Methanobacterium thermoautotrophicum* (MthK) channels. However, it fails to modify gating of slo1-related channels (slo2, slo3) by Na^+/Cl^- or H^+/OH^- (Liu et al., 2013) nor the gating of slo1 itself by Mg^{2+}_i in absence of Ca^{2+} (Liu et al., 2008). A recent report, however, has shown that *in presence of Ca^{2+}* , Mg^{2+}_i modulates ethanol action on BK currents in cultured hippocampal neurons: this modulation is bimodal, as Mg^{2+}_i 200 μM ethanol decreases BK activity whereas activity increases as Mg^{2+}_i reaches 1 mM (Marrero et al., 2015). The study seems to underscore that Mg^{2+} direct interaction with slo1 channels does not influence ethanol action, as the reported bimodal effect of ethanol as function of Mg^{2+}_i levels only occurs in presence of a preserved intracellular actin: destabilization of actin at the cytoplasmic side of the patch results in Mg^{2+}_i -independent activation of BK currents by ethanol. In contrast, presence of an actin stabilizer leads to ethanol-induced BK current inhibition even at 1 mM Mg^{2+}_i (Marrero et al., 2015). Mg^{2+}_i modulation of ethanol action is of particular relevance as Mg^{2+}_i has been reported to be reduced in both experimental systems exposed to ethanol and in intracellular compartments and serum of alcoholics (Altura & Altura, 1999; Babu, Cheng, Zhang, Altura, & Altura, 1999; Brown, Ilg, Chen, & Ren, 2002; W. Li et al., 2001; Poikolainen & Alho, 2008; Romani, 2008).

Slo1 protein isoforms—Ethanol modulation of BK channel activity has been consistently reported *in vitro* using highly simplified model systems (reviewed in Brodie et al., 2007; Dopico et al., 2014; Mulholland et al., 2009). In a most reductionist approach, ethanol-induced channel activation has been documented after reconstituting hslol (cloned from human brain) proteins in an artificial planar bilayer made of two phospholipid species: phosphoglyceride, i.e., 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl phosphatidylserine (POPS) (Crowley et al., 2003) indicating that this very simple proteo-lipid system must contain an ethanol sensor(s) that leads to modification of BK current. Indeed, a combination of electrophysiological studies on slo1 and related channels, including its prokaryotic analog MthK and chimeric slo channels (Liu et al., 2013), followed by alcohol probing of point mutants, and MM and MDs data based on

crystallography of the slo1 CTD led to the identification of a protein pocket of discrete dimensions in the slo1 protein that docks ethanol leading to increased channel activity (Bukiya et al., 2014). The ethanol pocket (Fig. 1) involves: 1) Lys 361 forming hydrogen-bond with ethanol; 2) net positive charge provided by R514 near the ethanol-Lys 361 interaction; 3) Glu 354, Ser 357 and Asn 358 allowing ethanol access to its Lys 361 bonding partner. Interestingly, the T352I substitution abolishes both ethanol induced channel activation and resistance to ethanol modification of slo1-mediated behavior in *C. elegans* (Davis et al., 2014). MM shows that Thr 352 is near the identified ethanol pocket, yet it points away from the pocket itself (Bukiya and Dopico, unpublished). A role for this residue in controlling the ethanol response of BK channels has been recently discussed elsewhere (Dopico et al., 2014).

The ethanol site briefly described in the previous paragraph is highly conserved among species, from *C. elegans* and *D. melanogaster* to humans (Davis et al., 2014). However, not all BK channel-forming slo1 isoforms respond similarly to a first exposure to clinically relevant concentrations of ethanol. For instance, ALCOREX-slo1 channels, i.e., a slo1 splice variant that contains exon 29 (ALCOhol Regulated EXon) showed greatest alcohol sensitivity in both magnitude and persistence of potentiation by ethanol. However, INSERTLESS-slo1 channels, i.e., completely spliced-out variant (thus, having neither ALCOREX nor STREX exons) were potentiated by alcohol in a reduced a briefer fashion. Finally, STREX-slo1 channels, i.e., a splice variant that contains the so-called “stress-axis hormone-regulated exon”, were completely insensitive to alcohol (Pietrzykowski et al., 2008). In addition, bslo channels (cloned from bovine aortic smooth muscle) are inhibited rather than activated by 10–100 mM ethanol (Dopico, 2003). In the latter, however, the presence of a Ser residue targeted by phosphorylation in the S0–S1 cytosolic loop seems to determine this BK channel’s response to ethanol (see below subheading on *Phosphorylation/dephosphorylation*).

Channel regulatory β subunits—In most mammalian tissues, BK channel-forming slo1 proteins are tightly associated with regulatory proteins termed BK β subunits. These subunits are much smaller than slo1 proteins as they consist of two short intracellular ends and two transmembrane segments (TM) joined by an extracellular (EC) loop. Four isoforms have been characterized, each coded by a different gene (*KCNMB1-4*). BK β subunits are characterized by rather specific tissue expression, with β 1 and β 4 prevailing in smooth muscle cells and CNS neurons, respectively (Orio, Rojas, Ferreira, & Latorre, 2002). Remarkably, BK β expression introduces drastic changes in the BK channel phenotype, some of these changes suited to serve physiology in a cell-specific manner. For example, association of β 1 with slo1 subunits drastically increases the apparent Ca^{2+} -sensitivity of the channel complex, an action not exerted by β 4 (Behrens et al., 2000; Brenner, Jegla, Wickenden, Liu, & Aldrich, 2000; Kuntamallappanavar, Toro, & Dopico, 2014). This β 1 action allows smooth muscle BK channels to activate in response to physiological levels of Ca^{2+}_i reached near the BK channel during myocyte contraction (Perez, Bonev, & Nelson, 2001). Thus, BK channel activation renders STOCs, which negatively feedback onto depolarization-driven Ca^{2+}_i influx, limiting cell contraction and favoring smooth muscle relaxation. Indeed, physiological and pharmacological activators of BK channels that act via

recognition by distinct site mapped to $\beta 1$ TM2 serve as effective vasodilators both *in vitro* and *in vivo* (Bukiya, Singh, Parrill, & Dopico, 2011; Bukiya, Vaithianathan, Toro, & Dopico, 2009). Considering that $\beta 1$ drastically modifies Ca^{2+} -driven gating parameters of the BK complex and ethanol modulation of BK channels is coupled to Ca^{2+} -driven gating (see above), it is not surprising that co-expression of $\beta 1$ subunits exerts a powerful influence on ethanol modulation of BK current. Ethanol studies on slo1 (cbv1) and $\beta 1$ subunits cloned from rat cerebrovascular myocytes demonstrate $\beta 1$ subunits shift the ethanol-induced activation-to-inhibition “crossover” toward lower Ca^{2+}_i ($\sim 3 \mu\text{M}$) (Bukiya, Liu, et al., 2009). Consistent with its lack of modulation on the channel apparent Ca^{2+} -sensitivity, $\beta 4$ subunits fail to modify such activation-to-inhibition crossover in mslo1 (Liu et al., 2008). Thus, $\beta 1$ modification of ethanol action results in ethanol reduction of BK channel activity when evaluated at low μM Ca^{2+}_i (Bukiya, Liu, et al., 2009), an ethanol action reported with the native cerebrovascular channel (P. Liu et al., 2004). Eventually, intoxicating levels of ethanol (50 mM) constrict both rat and WT mouse cerebral arteries while failing to do so in arteries from *KCNMB1* knockout (K/O) mice (Bukiya, Liu, et al., 2009; Bukiya, Vaithianathan, et al., 2011).

Characterization of BK channel in nerve terminals vs. somata of rat supraoptic neurons revealed two distinct phenotypes displaying ethanol-sensitivity/charybdotoxin (Cbtx)-resistance and ethanol-resistance/Cbtx-sensitivity, respectively (Dopico, Chu, Lemos, & Treistman, 1999). This pharmacological profile could be attributed to differential expression of BK $\beta 4$ subunit between the different neuronal compartments, as heteromeric BK channels containing the neuronally-abundant $\beta 4$ subunit are resistant to the BK channel selective blocker Ibtx (Meera, Wallner, & Toro, 2000). Indeed, somata BK channels from supraoptic neurons were Ibtx-resistant (Wynne et al., 2009). In addition, analysis of somatic BK channel gating kinetics shows that they have fast activation kinetics, in contrast to the slow gating of neuro terminal channels. Finally, somatic channels are insensitive to ethanol, in contrary to terminal BK channels that are potentiated by the drug. It should be noted that based on biophysical and pharmacological properties of the somatic and neuro terminal BK channels and by immunohistochemical examination, authors confirmed the expression of $\beta 1$ and $\beta 4$ subunits in somatic and nerve terminal compartments, respectively. Thus, the differential ethanol response of BK channels in different domains of supraoptic neurons has been attributed to this selective distribution of $\beta 1$ and $\beta 4$ subunits in different neuronal compartments (Wynne et al., 2009).

In rat N. Acc MSN, single-channel recordings obtained from BK channels in the somata showed a phenotype similar to that of slo1 channels co-expressed with $\beta 4$ subunits while those in the dendritic domain of these neurons showed a phenotype similar to that of slo1 channels co-expressed with $\beta 1$. Remarkably, ethanol (10–50 mM) increased BK Po in MSN somata while failing to do so in their dendritic counterparts (G. E. Martin et al., 2008). Collectively, results from different excitable tissues (vascular smooth muscle, neurons and neuronal domains) point at β subunits as critical modulators of BK channel pharmacology, and acute ethanol sensitivity in particular. The role of BK β subunits in adaptative responses of BK channels to protracted/repeated ethanol exposure is discussed in the next section.

Post-translational modifications of BK subunits—Abundant literature documents that ethanol-modulation of BK currents is regulated by phosphorylation or dephosphorylation, with the channel *slo1* subunits being the likely targets of such post-translational modifications. In GH3 cells reported the possible contribution of phosphorylation process in ethanol action where ethanol-induced potentiation of BK channel activity is abolished by protein kinase C (PKC) inhibition and bolstered by phosphatase (PP) inhibitors (Jakab et al., 1997). In GH4/C1 and GH4/C1-STREX cells, ethanol-induced increase in BK steady-state activity was observed only in some membrane patches; authors attributed this variability to posttranslational modification of *slo1* proteins. Indeed, PKC blockers diminished ethanol potentiation of BK channel activity in GH4/C1 cells. However, PKC blockers had no effect on GH4/C1-STREX cells (Jakab et al., 2006; Jakab et al., 1997). Further investigation demonstrated that alcohol-mediated inhibition of STREX-containing BK channels is PKA-dependent. In the absence of PKA inhibitor (14–22 Amide), alcohol exposure decreased activity of BK-STREX channels. Block of BK-STREX phosphorylation by PKA caused the potentiation of the channel by alcohol (Brodie et al., 2007; Pietrzykowski et al., 2008).

As mentioned under “*Slo1 protein isoforms*”, *bslo1* channels are inhibited by 10–100 mM ethanol when studied in the same expression system and under the same conditions in which the drug evokes activation of *mslo1* channels (Dopico, 2003; P. Liu, Liu, Huang, Li, & Dopico, 2003). *Bslo1* protein distinctly includes a Thr residue in the S0–S1 cytosolic loop (*bslo* position 107). Incremental phosphorylation of channel subunits on this residue by Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) progressively increases channel *Po* and also gradually switches the channel’s ethanol responses from robust activation to inhibition (Liu, Asuncion-Chin, Liu, & Dopico, 2006). Thus, CaMKII phosphorylation of *bslo1* Thr107 seems to override ethanol allosteric coupling to channel activation by physiological levels of Ca^{2+}_i . It is important to note that Thr107 is not conserved in *mslo1*, *hslo1* or *cbv1*; all these isoforms contain non-phosphorylatable residues and are activated by ethanol at nM to low M Ca^{2+}_i (reviewed in Brodie et al., 2007; Dopico et al., 2014).

A recent investigation examining the influence of PKA, CaMKII and PP on ethanol actions on *slo1*± $\beta 4$ channels in HEK 293 cells and native BK channels in NAcc MSN has revealed that $\beta 4$ subunits drastically alter the effects of PKA, CaMKII and PP (Velázquez-Marrero, Seale, Treistman, & Martin, 2014). This study clearly demonstrates that PKA potentiates BK steady-state activity, an action that is completely blocked by $\beta 4$ subunit expression. In addition, in presence of this subunit, PKA modulation of the BK channel is not necessary for potentiation by alcohol. In contrast, homomeric BK channels are tonically inhibited by CaMKII. Regarding this CaMKII action, $\beta 4$ subunits play a subtle role, as they do not block CaMKII effect. The study also shows that in presence of CaMKIIN, a specific CaMKII inhibitor, *slo1*± $\beta 4$ channels develop ethanol tolerance; this type of tolerance was reported in *KCNMB4* K/O mouse but absent in WT ($\beta 4$ subunit-containing) BK channels (Martin et al., 2008). However, Velázquez-Marrero et al. (2014) document that co-expression of $\beta 4$ subunits, does not modulate ethanol action on homomeric BK channels, confirming previous data from recombinant *mslo1* channels (Liu et al., 2008). On the other hand, phosphatases inhibit homomeric *slo1* channels, an action that is reversed by $\beta 4$ subunit co-expression. In

turn, slo1 channel's rapid tolerance to ethanol is reversed by phosphatase inhibition (Velazquez-Marrero et al., 2014). The molecular mechanisms by which BK channel $\beta 1$ and $\beta 4$ participate in phosphorylation/dephosphorylation regulation of ethanol action on BK channels remain to be elucidated. The role of phosphorylation/dephosphorylation processes in BK channel adaptation to protracted/repeated ethanol exposure is discussed in a separate section below.

Lipid microenvironment of the BK channel complex—The role of membrane lipids in modification of ethanol's final effect on BK steady-state activity has been reported in many studies (reviewed by Bettinger & Davies, 2014; Dopico et al., 2014; Dopico, Bukiya, & Singh, 2012). Overall, bilayer thickness, lipid molecule 'effective shape', and presence of distinct lipid species in the bilayer all play critical roles in modulating ethanol action on BK Po.

Modification of bilayer thickness by changing the lipid acyl chain length affects the time course of the acute response to alcohol and can turn ethanol-induced activation into inhibition: while ethanol strongly potentiated hslol channels embedded in a thin bilayer, the alcohol inhibited these channels when probed in a thicker bilayer (Yuan, O'Connell, Wilson, Pietrzykowski, & Treistman, 2008). Insights on the mechanisms by which bilayer thickness affects ethanol modulation of BK function will become more accessible from our growing understanding of the structural bases of slo1 channel gating. In slo1 proteins, the linker that connects the S6 gate to the RCK domains forms a passive spring connected to the gating ring that is involved in Ca^{2+}_i -dependent activation (Niu, Qian, & Magleby, 2004). A simple mechanical model to explain ethanol modulation of channel function by bilayer thickness has been hypothesized: lateral stress (which tends to decrease Po) is counteracted by local hydrophobic mismatch between membrane lipids and the slo1 protein and forces generated by Ca^{2+} -binding to the gating-ring and communicated to the gate itself via the spring, which leads to increased Po in thick bilayers (Yuan, O'Connell, Jacob, Mason, & Treistman, 2007). In the presence of alcohol, Ca^{2+} -activation of BK channels appears reduced, as determined from the $V_{0.5}\text{-Ca}^{2+}$ plots (Liu et al., 2008; Bukiya et al., 2009). It is possible to speculate that ethanol reduced action in thicker bilayers results from ethanol impairing Ca^{2+} -binding (in particular at tens of μM Ca^{2+}) and/or the alcohol impairing coupling between Ca^{2+} -binding and the gate, probably *via* perturbation of S6-gate spring function.

Modulation of ethanol action on BK channel activity by the lipid "effective shape" is independent of lipid head net charge. Thus "type I" lipids (those with a polar head cross sectional area larger than the tail area) such as phosphatidylserine, introduce "positive monolayer curvature" and favor ethanol-induced activation of hslol channels following their reconstitution into planar lipid bilayers. In contrast, ethanol-induced potentiation of hslol channels is blunted by "type II" lipids (those with a polar head cross sectional area smaller than the hydrophobic tails/rings), such as phosphatidylglycerol and cholesterol (CLR) (Crowley et al., 2003; Crowley, Treistman, & Dopico, 2005). Remarkably, ethanol and cholesterol change channel dwell-times in opposite ways: while ethanol increases mean open time and decreases mean closed time (both actions leading to increased BK Po) cholesterol decreases mean open time and increases mean closed time. Although dwell times represent an aggregate reflection of multiple processes that underlie channel gating, the most

straightforward explanation for CLR functional antagonism on ethanol-induced BK current potentiation is that CLR and ethanol share a same target(s) that they modulate in opposite directions. Considering that ethanol can be conceptualized as a “type I” molecule, authors have speculated that the reduced ethanol activation of BK current in presence of CLR in a POPE/POPS bilayer was due to opposing effects of “type I” ethanol molecules and “type II” CLR molecules on bilayer physical properties (Crowley et al., 2003, 2005). Other possibilities cannot be excluded: CLR antagonism of slo1 channel potentiation by ethanol may be attributed to CLR making it more difficult for ethanol molecules to partition into the bilayer and thus reaching eventual channel subunit targets. This partitioning-lipid phase hypothesis, modification of bilayer physical properties by cholesterol and ethanol, and possible cholesterol-ethanol interaction at common BK protein target sites are discussed in detail elsewhere (Dopico et al., 2012).

Remarkably, BK channels made of channel-forming cbv1 and $\beta 1$ subunits in CLR-free bilayers show resistance to 50 mM ethanol. Inclusion of 23 mol% CLR into the lipid mixture results in ethanol-induced BK channel inhibition (Bukiya, Vaithianathan, et al., 2011). Collectively, studies from homomeric and heteromeric BK channels in lipid bilayers where CLR can be titrated show that CLR blunts ethanol-induced activation (homomeric slo1 channels) or turns channel refractoriness to inhibition by ethanol (slo1+ $\beta 1$ channels). Thus, ethanol seems to require cholesterol presence to inhibit $\beta 1$ subunit-containing BK channel. This conclusion obtained from experiments in artificial lipid bilayers seems is supported by data obtained in patches from rat and mouse cerebral artery myocytes that contained native (cbv1+ $\beta 1$) BK channel complexes. Ethanol-induced cerebral artery constriction that results from ethanol-induced BK channel inhibition is also ablated in rat and mouse arteries following cholesterol depletion (Bukiya, Vaithianathan, et al., 2011).

The molecular mechanism(s) underlying ethanol-CLR interactions on BK channels remain unclear. Remarkably, ethanol-CLR antagonism on slo1 channels could not be observed when CLR was substituted by its enantiomer, *ent*-CLR (C. Yuan, Chen, Covey, Johnston, & Treistman, 2011), suggesting that CLR modulation of ethanol action on BK channels involves specific CLR-protein interactions. Both ethanol-recognition (see above) and CLR-sensing sites have been mapped to the CTD of slo1 proteins, yet they do not overlap. CLR-sensing areas include seven cholesterol recognition amino acid consensus (CRAC) domains, with CRAC4 (the domain adjacent to the inner membrane leaflet where CLR is particularly abundant) playing a major role in the channel’s CLR sensitivity (Singh et al., 2012). How CLR-sensing sites and the ethanol-docking site described above may interact with each other upon respective ligand recognition remains unknown. Finally, it should be underscored that BK channels cluster in CLR-enriched membrane domains, e.g., “rafts” (reviewed in Dopico et al., 2012). It has been speculated that BK channels may redistribute in cell membranes upon chronic alcohol exposure (Crowley et al., 2003; C. Yuan et al., 2008). The role of lipids in BK channel plasticity in response to ethanol is reviewed in the next section.

Adaptations in BK channel/coding genes to protracted or repeated ethanol exposure: from molecular mechanisms to modification in ethanol-induced perturbation of behavior

Adaptation(s) in the response of a biological system that results in reduced response to a given stimulus is referred to as “tolerance”. When the stimulus is a drug (e.g., ethanol exposure) tolerance is usually associated with repeated exposure to that drug (Chandler, Harris, & Crews, 1998). Some individuals, however, can be relatively resistant or tolerant to ethanol compared to others when first exposed to the alcohol. Thus, from a time-frame point of view, several types of tolerance to ethanol have been distinguished: “innate/intrinsic” tolerance, also termed “low level of response to ethanol” (Schuckit, 2000), can be described as a reduced response to ethanol from an alcohol-naïve system, i.e., the response of the individual is significantly smaller than that of the normal population. The terms “acute” or “within session” tolerance have also been used to describe a reduced response to ethanol that develops within a single exposure or single episode of intoxication (in case of behavioral evaluation) and typically occurs within minutes of the initial exposure (Le, 1990; Tabakoff, Cornell, & Hoffman, 1986). In turn, development of tolerance during a second (or more) ethanol exposure 8–24 h. after the previous exposure has been traditionally defined as “rapid tolerance” (Khanna, Chau, & Shah, 1996). Finally, “chronic tolerance” has been applied to a reduced response to ethanol following repeated or protracted exposure to ethanol over a period of days or weeks (Khanna et al., 1996). Disregarding time-frame, the two major “branches” of Pharmacology, Pharmacokinetics and Pharmacodynamics, offer several explanations for developing tolerance to ethanol. The former involves modifications in absorption, distribution, metabolism and/or excretion (ADME) of ethanol by the organism. To our knowledge, there are no studies documenting that specific changes in ADME at the organism level contribute to reduced response of BK channels to ethanol. On the other hand, “Pharmacodynamics-driven” tolerance involves adaptation(s) occurring in one or (usually for a ligand with simple chemical structure such as ethanol) many “sites of action” whether at genetic, epigenetic or post-translational levels. In this section, we will briefly summarize the literature dealing with “Pharmacodynamics-based” tolerance in the BK channel-ethanol interaction, expanding from subcellular to behavioral studies. The latter have been recently described in detail elsewhere (Bettinger & Davies, 2014). It should be pointed out from the beginning that the contribution of memory, operant, associative or other forms of learning (Kalant, 1998) to tolerance in alcohol-BK channel interactions, remains to be established. In addition to the time-based terminology that categorizes tolerance, we will use the term “molecular tolerance” (Treisman & Martin, 2009) when plasticity in the interactions between BK channel proteins/coding gene(s) and ethanol have been resolved at the subcellular level but several macromolecules seem to participate in such adaptation and/or the role of a unique ethanol-macromolecule interaction has not yet been determined. Within the concept of “molecular tolerance”, if a given macromolecule that had responded to ethanol first application was unequivocally identified as mediator or final effector in the ethanol adaptive response, the “desensitization” should be applied (Dopico & Lovinger, 2009). Whichever the time frame and level of resolution, identification of the mechanisms that underlie tolerance/resistance to ethanol exposure acquires particular relevance as

tolerance to ethanol in humans has advanced as a predictor for the likelihood of developing alcoholism (Schuckit, 2000; Schuckit, Klein, Twitchell, & Smith, 1994).

Critical advances in identifying targets and mechanisms contributing to development and/or maintenance of behavioral tolerance to ethanol have been initially limited to invertebrate model systems, such as *D. melanogaster* and *C. elegans*, as they are amenable to wide genetic manipulation, characterized by short-generation times, and present CNS neurocircuits that are simpler and therefore, are better characterized than those of mammals. Rapid tolerance measured as reduction in sedative phase following single previous sedation with ethanol vapor has been documented in WT *D. melanogaster* but not in *Slowpoke* (ortholog to *Slo1* or *KCNMA1* in mammals) null flies (Cowmeadow et al., 2005). In these experiments, flies were sedated by ethanol vapor within 20 minutes of vapor introduction. After full recovery in fresh air, vapor was delivered again. After switching ethanol vapor to fresh air again, the number of flies as percentage of all population recovering from sedation every minute was used to characterize the tolerance. Remarkably, block of slowpoke expression in the nervous system prevented development of rapid tolerance (Cowmeadow et al., 2005). Increased BK channel function allows for shorter neuron refractory periods and thus, enhanced neuronal excitability and faster recovery from ethanol sedation (Ghezzi et al., 2010). More recent work in *Drosophila* unveiled DNA elements responsible for modulation of BK channel expression following alcohol exposure (Li, Ghezzi, Pohl, Bohm, & Atkinson, 2013). In this set of experiments, flies were exposed to benzyl alcohol-coated vials until fully sedated within minutes. Flies recovered from sedation were exposed to benzyl alcohol again in 24 hours. Results show that the increased BK channel expression in neurons of benzyl alcohol-exposed flies is mediated by a drug-responsive element: genomic deletion of 6b DNA element results in a phenotype with increased duration of tolerance and symptoms of withdrawal. However, this deletion does not alter other *Slo*-dependent behaviors (Li et al., 2013). In addition, cyclic AMP response element-binding (CREB) proteins have been linked to *Drosophila* tolerance to benzyl alcohol: benzyl alcohol sedation alters expression of dCREB-A and dCREB2-b genes resulting in increased production of positively acting and reduced presence of negatively acting CREB isoforms. Moreover, loss-of-function allele of dCREB2 ablates the increase in BK channel expression and eventual tolerance following benzyl alcohol exposure (Y. Wang, Ghezzi, Yin, & Atkinson, 2009).

In mammals, chronic tolerance in response to 20–40 day-long ethanol intake (rendering 30 mM BAL) has been measured as a reduction in both activation of BK channels and inhibition of vasopressin and oxytocin release by rat dissociated neurohypophysial terminals in response to acute ethanol challenge (Knott et al., 2000; Knott et al., 2002). Authors attributed plasticity in BK channel response following chronic alcohol exposure to decreased BK current density (Knott et al., 2002). Also in the rat species, combination of patch-clamp electrophysiology and immunolabeling against BK channel-forming slo1 subunits in hypothalamic–neurohypophysial explants shows two components of tolerance. The first is given by the decreased potentiation of BK channel current by ethanol. This component of tolerance was evident at 30 min of prolonged ethanol exposure and persisted throughout 24 hours of exposure. The second component of tolerance consisted of decreased current density, this component having a delayed time course: it was first seen several hours after the initiation of prolonged exposure to ethanol. Regarding this second component, confocal

microscopy showed reduction in BK channel protein clustering in the plasma membrane during ethanol exposure (Pietrzykowski et al., 2004). A recent study in primary cultures of rat hippocampal neurons shows a complex pattern in BK channel membrane expression in response to 25 mM ethanol applied for 10 min, 3, 6, and 24 hours: total internal fluorescence (TIRF) microscopy shows that perimembrane channel expression increases during 10 min and 3 hours applications, decreases after 6 hours, and returns to pre-ethanol levels after a day of ethanol exposure. In contrast, this recovery was not observed when BK perimembrane expression was determined after a 6 hours-long ethanol exposure followed by an 18 hours-long ethanol withdrawal period. On the other hand, electrophysiological data did not parallel the TIRF data at every time point, as current density is actually decreased following a 3 hours-long ethanol exposure. Thus, differences in BK channel membrane clustering (Palacio et al., 2015) and/or receptor desensitization likely contribute to the overall adaptive response of BK current density to protracted ethanol exposure.

In addition to internalization of membrane BK channels as a mechanism underlying rapid tolerance to ethanol, alcohol-driven changes in the brain BK channel splice variant profile have been reported, this arrangement being controlled by microRNAs. Specifically, miR-9 mediates destabilization of BK mRNAs that contain 3'UTRs with a miR-9 recognition element, which leads to a switch between alternatively spliced BK mRNA variants. Thus, drop in the highly alcohol-sensitive ALCOREX/INSERTLESS and increase in the alcohol-resistant INSERTLESS/STREX variant are detected (Pietrzykowski et al., 2008). Moreover, data from mouse cultured and acutely dissociated striatal neurons revealed that longer exposure times to alcohol required longer recovery for BK channel ethanol sensitivity. In particular, BK channel alcohol sensitivity after 6 h exposure to 20 mM ethanol was diminished beyond 24 hours after withdrawal (Velazquez-Marrero et al., 2014). In contrast, after a 1 or 3 hours-long exposure to ethanol, BK channel alcohol sensitivity had significantly recovered after 4 hours. This time-controlled tolerance to alcohol exposure was paralleled by changes in BK channel isoform profile with increased mRNA levels of the alcohol-insensitive stress axis-regulated (STREX) splice variant (Velazquez-Marrero et al., 2011).

Subunit composition of BK channel heteromers is another important determinant of the two components of ethanol tolerance identified by Pietrzykowski et al. (2004) (see above). Patch-clamp data from hslol channels expressed in HEK cells indicate that ethanol-driven downregulation of BK current density is found in homomeric hslol and heteromeric hslol+ β 4, yet not in hslol+ β 1 channel complexes. Thus, β 1 subunits decrease the “second component” of BK channel tolerance to acute challenge by ethanol. In contrast, “the first component” of alcohol tolerance has been observed in channels having all three subunit compositions, with homomeric slo1 channels displaying the greatest tolerance. Thus, β 1 and β 4 subunits are similarly efficacious in blunting the first component of acute tolerance (Feinberg-Zadek, Martin, & Treistman, 2008). In a different experimental setting of protracted (8 min-long), but not repeated challenge with ethanol (50 mM), β 4 subunit-containing BK complexes exhibit less tolerance to acute ethanol challenge than homomeric channels (consisting of only alpha subunits) (G. E. Martin et al., 2008). Consistently, the BK channel in freshly isolated mouse striatal MSNs, which contains β 4 subunits, exhibits little tolerance to acute ethanol challenge. In contrast, striatal MSNs neurons from *KCNMB4* K/O

mouse do not exhibit ethanol tolerance (G. E. Martin et al., 2008). A recent study using similar experimental patch-clamp paradigm points at ability of $\beta 4$ subunit to influence regulation of BK channels by PKA and CaMKII as a mechanism for $\beta 4$ subunit-mediated modulation of tolerance. Interestingly, slo1 channel's rapid tolerance to ethanol is reversed following PP inhibition. In addition, slo1+ $\beta 4$ channels under pharmacological CaMKII inhibition develop ethanol tolerance (Velazquez-Marrero et al., 2014). The specific mechanisms engaged in the interplay between slo1, $\beta 1$ and $\beta 4$ subunits, kinases, phosphatases and alcohol remain speculation. Considering that $\beta 4$ subunit contains several phosphorylation substrate motifs, it is highly likely that phosphorylation of $\beta 4$ subunits participates in ethanol tolerance of BK channels. It is also possible that $\beta 4$ modulates adaptive responses of slo1 to ethanol *via* protein-protein interaction independently of phosphorylation processes.

The data described above from subcellular resolution experiments have been complemented by behavioral studies in rodents. Constitutive deficiency in BK $\beta 1$ or $\beta 4$ subunits in mice does not alter BAL, nor sensitivity to ataxia, sedation and hypothermia evoked by acute ethanol administration. BK $\beta 1$ deletion reduces chronic tolerance (after repeated alcohol exposure) to sedation and hypothermia while BK $\beta 4$ deletion does not affect these adaptations to chronic ethanol exposure. The lack of effect of BK $\beta 4$ deletion in this work is somewhat surprising in light of the work by Martin et al. (2008) described above. Authors speculate on two possibilities: 1) the intoxication conditions used in their study (4 cycles of 16 hours-long vapor) could have been “too harsh”, rendering maximal tolerance and thus failing to capture the intermediate points of tolerance development at which role of $\beta 4$ subunit could have been most prominent; 2) the role of $\beta 4$ subunits in development of tolerance in MSNs may be different from the role of these subunits in tolerance to sedation and hypothermia (Kreifeldt, Cates-Gatto, Roberts, & Contet, 2015). Interestingly, the hyperexcitable state (determined by handling-induced convulsions) associated with ethanol withdrawal is accelerated by the absence of BK $\beta 1$ subunits while delayed by the absence of BK $\beta 4$ (Kreifeldt et al., 2015). These findings bolster the case for a critical role of BK channel subunit composition in ethanol tolerance and resulting behavior.

Genetic ablation of *KCNMB1* or *KCNMB4* not only affects neurological perturbations linked to protracted or repeated ethanol administration but also modulates escalation of alcohol drinking in mice. Remarkably, genetic deletion of $\beta 1$ or $\beta 4$ regulatory subunits (on C57BL/6J background) altered voluntary ethanol drinking in alcohol-dependent mice while failing to do so in non-dependent animals. Indeed, whether mice were subjected to a continuous two-bottle choice followed by intermittent two-bottle choice paradigm, or trained to drink ethanol in a limited-access two-bottle choice paradigm, baseline alcohol drinking between WT, *KCNMB1* K/O or *KCNMB4* K/O were similar. In contrast, *KCNMB1* K/O and *KCNMB4* K/O respectively displayed an increased and decreased escalation of ethanol drinking during withdrawal from chronic intermittent ethanol administration (Kreifeldt et al., 2015). Along the authors' arguments, it is possible to speculate that $\beta 1$ -induced blunting of neuronal BK channel's responses to ethanol and $\beta 4$ -induced suppression of BK channel's acute tolerance to protracted ethanol exposure (see above) lead to the aforementioned changes in alcohol escalation in dependent mice, yet the mechanisms linking in vitro observations to changes in drinking escalation need to be established.

In summary, while details of β subunit-type modulation of alcohol tolerance are still under investigation, there is little doubt that BK subunits play a critical role in both acute and chronic behavioral tolerance to ethanol. Considering that BK $\beta 4$ subunits are the prevalent β subunit type in the brain (Brenner et al., 2000) and that control development of alcohol tolerance at molecular, cellular, and organ levels, BK $\beta 4$ subunit may be proposed as a novel therapeutic target for alcoholism.

Although a major component underlying acute tolerance to ethanol seems to be restricted to the BK protein complex, including channel subunit ability to disrupt modulation of channel function by kinases and phosphatases (see above), this type of tolerance has been shown to be conditioned by membrane lipids. In *C. elegans*, regulation of acute tolerance by lipid environment has been studied *in vivo*. Worms that have been starved of exogenous cholesterol (which is necessary for their normal physiology), do not exhibit acute tolerance when placed into media containing 400 mM ethanol, which renders 22 mM ethanol from the worm's lysate; (Davies et al., 2003). Thus, alcohol tolerance seems to require cholesterol presence (Bettinger, Leung, Bolling, Goldsmith, & Davies, 2012). In addition, *lips-7(ok3110)* worms, which carry a mutation in the triacylglycerol pathway lipase LIPS-7, are resistant to high dosage of ethanol in the environment, and are fast developers of acute (within 30 minutes) tolerance (Bettinger et al., 2012).

Data obtained in *C. elegans* are in agreement with *in vitro* data from *hslo1* channel incorporated into the planar lipid bilayers. In this simplified setting, BK channels exhibit acute (within 10 minutes) tolerance to channel activation in response to 50 mM ethanol. An initial ethanol-induced BK channel activation (observed within 5 minutes) is favored by thin lipid bilayers, yet this activation vanishes and BK channel inhibition appears as bilayers consist of lipid species that form thicker bilayers (C. Yuan et al., 2008). Moreover, a sustained response to ethanol ("alcohol tolerance") is also modified by bilayer thickness: in thicker bilayers, a protracted ethanol exposure of 5–8 minutes leads to channel inhibition, the latter not being observed in thin bilayers (C. Yuan et al., 2008). In addition to possible pharmacodynamic explanations, authors hypothesized that modification of the channel's lipid environment might selectively alter the access of ethanol molecules to its site(s) in the channel protein, leading to differential ethanol effects on BK Po (C. Yuan et al., 2008). Collectively, *in vivo* findings from *C. elegans* and *in vitro* findings with BK channels from human brain reconstituted into lipid bilayers of controlled proteolipid composition indicate that membrane lipids play an important role in shaping up the adaptive responses of *slo1* channels to protracted ethanol administration.

Conclusions

During the last two decades, several studies have demonstrated that *in vitro* exposure of BK channels to ethanol concentrations reached in blood during alcohol intoxication in humans disrupts physiology. Recently, an ethanol-recognition pocket of discrete dimensions have been mapped to the BK channel-forming *slo1* protein cloned from mouse brain, this region being highly conserved across species. The importance of this region in acute alcohol intoxication has been determined by evaluating *slo1* mutants in *C. elegans*. It is also clearly established that the final response of BK channels to ethanol action from alcohol-naïve

systems, however, is determined by a wide variety of molecules that impact on BK channels. Moreover, the role of some of these factors in adaptative responses of BK channel proteins/coding genes to protracted and/or repeated ethanol exposure is beginning to emerge. Thus, tolerance to ethanol exposure seems to involve modifications at posttranslational, epigenetic and genetic levels. In particular, the role of the *Slo* gene in tolerance to alcohol intoxication has been determined in *D. melanogaster*. Very recent studies in rodents underscore the impact of genetic manipulation of BK channel regulatory β subunits in alcohol-driven behaviors, including ethanol drinking in alcohol-dependent mice. Whether memory or other mental processes involved in learning contribute to tolerance in the alcohol-BK channel interaction remains unknown. Likewise, the contribution of specific pharmacokinetic processes to individual tolerance to ethanol effects on BK channels remains to be explored. While major unknowns in the ethanol-BK channel field lie ahead, the importance of this area of investigation cannot be overstated. On one hand, identification of specific ethanol-recognition sites in effectors of ethanol action on BK channels is a critical first step for designing agents that could counteract modification of physiology elicited by ethanol action on BK channels. On the other, identification of mechanisms determining or, at least, contributing to BK channel-ethanol tolerance is of paramount importance as tolerance to alcohol has been proposed as a predictor of risk for developing alcoholism.

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Figure 1.

Ethanol-sensing site in the BK channel-forming slo1 protein cytosolic tail domain (CTD). (A) Location of the ethanol-sensing site in the slo1 CTD; the CTD is modeled based on the crystallographic structure obtained in Ca^{2+} -containing solution (see main text). The ethanol carbon chain is shown in pink; oxygen is in red. Right top insert shows the ethanol-sensing site provide by residues 354–367 where Lys361 hydrogen bonds (dotted line) with ethanol. Nitrogen is in blue; hydrogen is hidden to improve display. (B) The ethanol-slo1 CTD model based on the crystallographic structure obtained in *absence of Ca^{2+}* (see main text) shows substantial perturbations within the ethanol-sensing site: repositioning of Lys361-containing helix; obstructed ethanol's access to the site due to the movement of Met909; displacement of Arg514 away from the ethanol-sensing area. Key elements in ethanol-sensing pocket are color-coded as in (A). However, Lys361, Met909 and Arg514, which experience the bigger perturbations by the absence of Ca^{2+} ; their location under this condition are shown in black and underlined. With modifications, from Bukiya et al. (2014).

Figure 2.

Role of the ethanol-docking slo1 CTD site in long-chain alkanol's effects on slo1 currents. Poses of n-alkanols on the site surface map show that slo1 activating n-alkanols, such as ethanol, propanol, butanol, heptanol and hexanol fit within the ethanol-sensing pocket while ineffective n-alkanols, such as octanol and nonanol do not (see main text). Ethanol carbon chain is in pink; other activating n-alkanols are shown in yellow whereas ineffective n-alkanols are in orange. In all cases, oxygen is in red, nitrogen is in blue. Hydrogen bonds between hydroxyl groups of alkanols and Lys361 are presented as dotted lines. This figure is based on previously published material by the authors (Bukiya et al., 2014).

Figure 3.

Cartoon illustrating our current interpretation of ethanol modulation of slo1 channel steady-state activity (P_o). Slo1 channel gating is basically presented as the integration of three allosterically-coupled equilibria: channel close (C)—open (O) transitions in absence of voltage or Ca^{2+}_i with “Lo” being the equilibrium constant; transition between resting (R)—activated (A) stages of the voltage sensor, with J as the equilibrium constant, and Ca^{2+}_i -free— Ca^{2+}_i -bound channel, with K being the equilibrium constant. Allosteric coupling factors between equilibria are given by C, D and E, following Horrigan & Aldrich 2002. Ethanol cannot modify slo1 P_o in absence of Ca^{2+}_i or activating voltage (i.e., ethanol does not modify Lo) (Liu et al. 2008). While the ethanol-sensitivity of J remains undetermined, crude parameters of voltage-sensing seem to indicate that voltage-sensing is not altered by ethanol. In contrast, ethanol modifies K, leading to increase or decrease in P_o dependent upon Ca^{2+}_i levels (Liu et al., 2008; Bukiya et al., 2009). Ethanol-sensitive and -resistant equilibria are given in red and light green boxes, respectively. Questions marks underscore that the alcohol sensitivity of key gating parameters in this allosteric model remains unexplored.

Table 1

effects of brief exposure (typically <5 min) to clinically relevant concentrations of ethanol (100 mM) on native BK channel function in alcohol-naïve systems*.

System	V-clamp method	Ethanol (mM)	Effect on channel activity/current	Likely consequence on tissue physiology	References
<i>Neuroendocrine cells and neurons</i>					
Rat GH3 and GH4/C1 pituitary cell lines	O/O, C/A patches	1–300	Potentiation	Inhibition of growth hormone release	Jakab et al., 1997; Handelechner et al., 2013
Rat supraoptic /paraventricular neurons (nerve terminals)	I/O patch, conventional WCR	10–100	Potentiation	Inhibition of vasopressin or oxytocin release	Dopico et al., 1995; 1996; Knott et al., 2002; Pietrzykowski et al., 2004; Wynne et al., 2009
Rat supraoptic /paraventricular neurons (somata)	I/O patch	25–100	Refractoriness	N/A	Dopico et al., 1999; Wynne et al., 2009
Rat primary sensory dorsal root ganglia neurons	O/O patch	40–80	Potentiation	AP shortening, increase in refractory period with decreased firing rate	Gruß et al., 2001
Rat NAcc neurons (somata)	I/O patch	1–100	Potentiation	AP shortening; reduced neuronal firing	Martin et al., 2004
Rat NAcc neurons (dendrites)	I/O patch	10, 50	Refractoriness	N/A	Martin et al., 2004
Mouse striatal MSN	C/A single-cell patch, WCR	50	Potentiation	AP shortening?; reduced neuronal firing	Martin et al., 2008
<i>C. elegans</i> type IV DA CEP neurons; also interneurons	WCR, I/O patch	400 (22), 500(29)**	Potentiation	Disruption of locomotion, egg laying	Davies et al., 2003
<i>Other cell types</i>					
Human umbilical vein endothelial cells in culture	I/O patch	10–50	Potentiation	Increased endothelial proliferation and NO• production	Kuhlmann et al., 2004
Bovine aortic smooth muscle	Planar lipid bilayer	5–80	Inhibition	Aortic constriction	Walters et al., 2000
Rat cerebral artery myocytes	I/O, O/O patches, PP WCR	3–200	Inhibition	Cerebral artery constriction	Liu et al., 2004; Bukiya et al., 2011
Mouse cerebral artery myocytes	I/O patch	50 mM	Inhibition	Cerebral artery constriction	Bukiya et al., 2009; 2011

* **Note1:** the listing *excludes* cell/animals where genes/transcripts encoding for BK channel subunits, whether channel-forming or regulatory, have been deleted, overexpressed or engineered (see main text).

** **Note2:** in this case, worms were exposed to ethanol-containing bath for 22 min, i.e., more than what is considered a typical “acute/brief” application. However, pharmacokinetic considerations should be taken into account as ethanol does not seem to readily cross the *C. elegans* cuticle. This is reflected by the very different external (bath) and corresponding internal (from worm lysates) ethanol concentrations, the latter given in parenthesis in this table.

Abbreviations: AP, action potential; C/A, cell-attached; DA CEP, dopaminergic cephalic sensilla; I/O, inside-out; MSN, medium spiny neurons; NAcc, nucleus accumbens; O/O, outside-out; PP, perforated patch, macroscopic current recording; WCR, standard whole-cell, macroscopic current recording.

Table II

factors that contribute to changes in the ethanol response of BK channels/BK subunit-coding genes following protracted or repeated ethanol administration.

<i>System</i>	<i>Type of ethanol administration</i>	<i>Factor(s), variable(s) or parameter(s)</i>	<i>Consequence on BK current and/or tissue response</i>	<i>References</i>
BK in HEK293 cells; lipid bilayer	50 mM ethanol for 15 min	Changes in bilayer bulk lipid composition	Loss of BK channel activation by acute ethanol	Yuan et al., 2008
HEK293 cells	Two 2 min-long exposures to 25 mM ethanol separated by 10 min wash. Also: 25 mM ethanol for 24 hr	Steady-state activity and current density (amplitude/capacitance) in BK channel heteromers	Rapid tolerance is robust in homomeric slo1 and in slo1+ β 4. Both constructs and slo1+ β 1 are all ethanol-tolerant after 24 hr ethanol.	Feinberg-Zadek et al., 2008
Rat cultured striatal neurons	Vapor on cell culture dish (20 mM ethanol)	Measurement (PCR) of slo1 splice variants; BK steady-state activity	Major drops in Alcorex and major increase in Strex; ethanol potentiation of current is greatly reduced	Pietrzykowski et al., 2008
Striatal neurons of C57BL/6J mice	<i>In vitro</i> exposure to 20 mM ethanol for 1, 3 or 6 hr	Slo1 transcripts	6 hr-long ethanol exposure lead to increased STREX and delayed recovery from tolerance	Velázquez-Marrero et al., 2011
Rat primary hippocampal neuronal cultures	25 mM ethanol applied for 10 min, 3, 6 and 24 hr	BK channel current, BK membrane expression and clustering (TIRF microscopy)	Successive increases and decreases in BK membrane expression and reduction in active channels after 3 and 6 hours of ethanol exposure	Palacio et al., 2015
Hypothalamic–neurohypophysial explants (rat)	Ethanol vapor on culture (20 mM). Exposure times of 0.5, 1, 3, 6 and 24 hr	Ion current response and channel membrane distribution and internalization	Reduced BK potentiation by ethanol within several min of exposure; BK internalization during hours-long exposure	Pietrzykowski et al., 2004
Isolated rat neurohypophysial terminals	Ethanol-rich diet for 20–40 days (30 mM BAL)	Peptide release (Radioimmunoassay)	Vasopressin release blunt by 10 mM ethanol is reduced in rats on ethanol-rich diet	Knott et al., 2000

<i>System</i>	<i>Type of ethanol administration</i>	<i>Factor(s), variable(s) or parameter(s)</i>	<i>Consequence on BK current and/or tissue response</i>	<i>References</i>
Isolated rat neurohypophysial terminals	Ethanol-rich diet for 3–4 wks (30–35 mM BAL by day 24 on diet)	BK current density (current amplitude/capacitance) and peptide release (radioimmunoassay)	Current potentiation and oxytocin release inhibition by ethanol are blunted in the chronic alcohol-diet group	Knott et al., 2002
Mouse striatal medium spiny neurons	<i>In vitro</i> : 50 mM ethanol on neuron/slice for 8 min. <i>In vivo</i> (DID): 10% ethanol bottles for 2 hr	BK current in isolated neurons, spike patterning in brain slices, motor activity and ethanol consumption (restricted access)	BK channels from WT mice do not exhibit tolerance while $\beta 4$ KO do. PKA, CAMKII, PP are involved. Rapid tolerance to ethanol locomotor effects develops in $\beta 4$ KO but not in WT. $\beta 4$ KO drink more than WT	Martin et al., 2008; Velázquez-Marrero et al., 2014
BK $\beta 1$, BK $\beta 4$ -deficient mice	4 routes (16 hr ethanol vapor/4 hr air); before each route: ethanol load (1.5 g/kg, i.p.); 43 mM BAL maintained with ethanol vapor	Motor coordination, righting reflex, body temperature, handling induced convulsions after ethanol withdrawal	Appearance of hyperexcitable state driven by ethanol withdrawal is accelerated in BK $\beta 1$ KO while delayed in BK $\beta 4$ KO	Kreifeldt et al., 2015
<i>Drosophila melanogaster</i>	Ethanol vapor twice; 4 hs between sedations	<i>Slowpoke</i> gene expression in the nervous system	Decreased time for ethanol-induced sedation	Cowmeadow et al., 2005
<i>Drosophila melanogaster</i>	Double exposure to benzyl alcohol-coated vials; 24 hr in-between	CREB transcription factor-stimulated gene expression/reduced CREB repressor	Rapid tolerance to sedation-induced <i>Slo</i> gene expression	Wang et al., 2009
<i>Drosophila melanogaster</i>	Benzyl alcohol-coated vials given 1,4,7,14,21 or 28 days after first exposure	Replacement of 6b element with loxP site in <i>Slo</i> transcription control area	Tolerance to benzyl alcohol even 28 days after first sedation episode	Li et al., 2013
<i>Caenorhabditis elegans</i>	400 mM ethanol in media for 30 min (<25 mM in worm tissue)	Cholesterol starvation, manipulation of triacylglycerol levels; locomotion	Decreased ability to develop fast tolerance as measured by locomotion assays	Bettinger et al., 2012

Abbreviations: BAL, blood alcohol level; CaMKII, Ca^{2+} -Calmodulin kinase II; PKA, protein kinase A; PP, phosphatase; TIRF, total internal reflection fluorescence