Published in final edited form as:

Nat Neurosci. 2006 January ; 9(1): 41–49. doi:10.1038/nn1602.

CaM kinase II phosphorylation of slo Thr107 regulates activity and ethanol responses of BK channels

Jianxi Liu, Maria Asuncion-Chin, Pengchong Liu, and Alejandro M Dopico

Department of Pharmacology, University of Tennessee Health Science Center, 874 Union Avenue, Memphis, Tennessee 38163, USA

Abstract

High-conductance, Ca^{2+} -activated and voltage-gated (BK) channels set neuronal firing. They are almost universally activated by alcohol, leading to reduced neuronal excitability and neuropeptide release and to motor intoxication. However, several BK channels are inhibited by alcohol, and most other voltage-gated K^{+} channels are refractory to drug action. BK channels are homotetramers (encoded by *Slo1*) that possess a unique transmembrane segment (S0), leading to a cytosolic S0–S1 loop. We identified Thr107 of bovine slo (bslo) in this loop as a critical residue that determines BK channel responses to alcohol. In addition, the activity of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) in the cell controlled channel activity and alcohol modulation. Incremental CaMKII-mediated phosphorylation of Thr107 in the BK tetramer progressively increased channel activity and gradually switched the channel alcohol responses from robust activation to inhibition. Thus, CaMKII phosphorylation of slo Thr107 works as a ‘molecular dimmer switch’ that could mediate tolerance to alcohol, a form of neuronal plasticity.

High-conductance Ca^{2+} -activated K^{+} (BK) channels are key to various physiological processes¹. In most tissues, BK channels consist of pore-forming (α , encoded by *Slo1* or *KCNMA1*) and accessory (β_{1-4}) subunits². The role of BK channels in a wide variety of processes is primarily ensured by slo isoforms and their post-translational modifications, which adjust the channels’ biophysical characteristics to specific cell processes³.

Neuronal BK channels participate in repolarization and fast after-hyperpolarization of action potentials, setting neuron firing properties⁴. It has been speculated that a drug-induced increase in Ca^{2+} -activated K^{+} currents might contribute to the depression of central neurons by ethanol and by sedatives and/or hypnotics⁵. Indeed, acute exposure to intoxicating concentrations of ethanol potentiates BK channels in neurons and neuroendocrine cells⁶, decreasing excitability⁷ and neuropeptide release and producing eventual diuresis⁸. In addition, ethanol potentiation of neuronal BK channels underlies alcohol-induced motor incoordination in *Caenorhabditis elegans*⁹.

Ethanol potentiation of BK channels, however, is not universal. Ethanol concentrations that activate neuronal BK channels inhibit aortic BK channels^{10,11}. Ethanol also inhibits BK currents in cerebral artery myocytes and thus causes cerebral artery constriction¹², a major mechanism underlying ischemic stroke linked to binge drinking¹³. Although several of the pathophysiological and behavioral consequences of ethanol modulation of BK channels have

Correspondence should be addressed to A.M.D. (adopico@utmem.edu).

Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions/>

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

been demonstrated, the molecular mechanisms that determine differential BK channel responses to ethanol remain unknown.

In isolated membrane patches from supraoptic neurons, ethanol potentiates nerve ending BK channels but not cell-body isochannels, suggesting that differential responses to ethanol are determined by differential targets and/or mechanisms located in the cell membrane¹⁴. Because *Slo1* encodes for BK channel-forming subunits, differential responses to ethanol should be attributed to slo isoforms, differential association with β -subunits or other proteins, and/or differential post-translational modification of the BK channel and associated proteins.

When expressed in *Xenopus laevis* oocytes, slo channels from mouse brain (mslo) are consistently and robustly activated by ethanol¹⁵. In the same system and under identical conditions, bslo channels from bovine aortic smooth muscle show heterogeneous ethanol responses: primarily inhibition and, much less frequently, refractoriness or even activation¹¹. Thus, regions that are not conserved between slo isoforms seem to determine the differential channel responses to ethanol. Indeed, we demonstrated that the 'core-linker' (S0–S8; Fig. 1a) is the primary determinant of BK channel responses to ethanol¹⁶. In addition, hslo (hbr1, from human brain) shows ethanol responses identical to those of mslo¹⁷. Primary sequence alignment of bslo, hslo (hbr1) and mslo (mbr5) shows a Thr107 in the bslo S0–S1 loop. In contrast, valine and alanine are found in equivalent positions in mslo and hslo, respectively (Fig. 1a,b). Thus, we hypothesized that Thr107 and its differential degrees of phosphorylation are responsible for the heterogeneity of BK channel responses to ethanol. Moreover, because mslo and hslo contain nonphosphorylatable residues in positions equivalent to Thr107, the lack of a phosphate in the S0–S1 loop might render BK channels activatable by ethanol.

Channel protein phosphorylation is a widespread mechanism that controls channel function¹⁸ and its modification by drugs, including ethanol^{19–21}. Multiple phosphorylation sites in slo are targeted by different protein kinases, resulting in either channel activation or inhibition²². CaMKII is an abundant neuronal and vascular smooth muscle protein^{23,24} that is necessary for the induction of long-term potentiation in mammalian central neurons and several learning protocols²⁵. As found for BK channels, CaMKII controls neuronal spike repolarization²⁶ and vascular smooth muscle tone²⁷. By considering (i) the key roles of BK channels and CaMKII in controlling common physiological processes and (ii) a sequence of five residues in the slo S0–S1 loop that represents a consensus site for CaMKII-phosphorylation²⁸, we determined the role of CaMKII phosphorylation of slo Thr107 in controlling the activity and ethanol responses of BK channels.

RESULTS

Mutation of slo Thr107 modifies channel ethanol responses

Primary sequence alignment of bslo and mslo showed that nonconserved regions occurred both in the S0–S1 loop and the S8–S9 linker. Considering that the vast majority of voltage-gated K^+ channels are refractory to modulation by clinically relevant concentrations of ethanol^{29,30} and that these channels lack the S0 segment, we mutated the threonine present in the S0–S1 loop that is not conserved between bslo and mslo.

Oocyte expression of bslo, mslo, bslo T107V (Fig. 2) or mslo V86T led to single-channel events that showed all the major characteristics of BK currents when recorded in inside-out (I/O) membrane patches using symmetric 130 mM K^+ solutions (Methods). First, at fixed free internal calcium concentrations ($[Ca^{2+}]_i$; 300 nM), the voltage–steady-state activity (that is, NP_o ; see Methods) relationship of both mutant channels was described by a Boltzmann function in which the plot of $\ln(NP_o)$ versus voltage was linear at low NP_o . Thus, the inverse of the

slope (k) was the potential needed to produce an e -fold change in NP_o (ref. 11): 13 ± 3 mV ($n = 6$) and 16 ± 3 mV ($n = 6$) for bslo T107V and mslo V86T, respectively. These values are similar ($P > 0.05$) and fall within the range reported for mslo, bslo and other slo channels^{11, 15, 17}. Second, the NP_o of both mutant channels obtained at any given voltage increased similarly with increases in $[Ca^{2+}]_i$ (data not shown). Finally, both mutant channels showed similar high unitary conductance: 225 ± 23 pS and 208 ± 18 pS ($P > 0.05$; $n = 6$) in symmetric 130 mM K^+ for bslo T107V and mslo V86T, respectively. These values are similar to those reported for mslo and bslo channels under equivalent conditions^{11, 15}. These similarities in k , Ca^{2+} activation and unitary conductance reflect the high identity that exists in the amino acid sequences of the voltage-sensing Ca^{2+} -bowl and pore regions across slo subunits²; further, they indicate that the mutations did not modify critical aspects of BK channel function.

Acute ethanol administration (100 mM) reversibly increased mslo channel NP_o in ten of ten I/O patches. In contrast, ethanol evoked varied responses in bslo channels: decreased activity in eight, increased activity in four and no change in four. After data from all samples passed a Kolmogorov-Smirnov normality test ($P > 0.1$), parametric analysis determined that the averaged ethanol responses of mslo and bslo ($196 \pm 16.67\%$ and $106 \pm 11.3\%$ of pre-ethanol controls) were different ($P < 0.05$), even when studied in the same batches of oocytes (Fig. 3a). These data confirm previous findings¹² and support the idea that regions that are not conserved between these slo subunits are major determinants of the differential BK channel responses to ethanol.

Ethanol readily and reversibly increased bslo T107V channel activity without modifying current amplitude (Fig. 2). Thus, drug action seems to be limited to modulation of channel gating. Ethanol, however, increased channel NP_o without modifying k : k was 13 ± 3 mV versus 12 ± 4 mV per e -fold change in NP_o in the presence and absence of ethanol, respectively ($n = 6$). Under steady-state conditions, an increase in NP_o may be determined by an increase in channel mean open time, a decrease in mean closed time or both. Ethanol did not change the mean open time, which was 2.6 ± 0.3 ms in the absence of ethanol versus 2.5 ± 0.2 ms in the presence of ethanol ($P > 0.05$; $n = 5$). Thus, ethanol potentiation of bslo T107V channels was caused by an increase in the frequency of channel openings (that is, decrease in channel mean closed time). These results are similar to those obtained with mslo¹⁵ and native BK channels in neurohypophyseal nerve endings⁴.

Ethanol-induced increases in bslo T107V NP_o were observed in 12 of 12 patches (Fig. 3b). Thus, for both bslo T107V and mslo, ethanol increased channel NP_o in all patches tested ($n = 22$). Not only the frequency of bslo T107V channel activity, but also its average potentiation ($247.8 \pm 15.3\%$ of controls), were similar to those found with mslo ($P > 0.05$) (Fig. 3a). In contrast, bslo T107V NP_o responses to ethanol were markedly different from bslo responses ($P < 0.001$). These data demonstrate that the single mutation of threonine to valine is sufficient to modify bslo channel responses to acute ethanol exposure.

In sharp contrast to its consistent activation of mslo and bslo T107V channels, ethanol had varied effects on mslo V86T channel activity: decreases (eight patches), increases (eight patches) and no change (three patches), a pattern similar to that of bslo channels (Fig. 3b). Furthermore, the average NP_o of mslo V86T in the presence of ethanol reached $123.63 \pm 13.44\%$ of that in controls, a value similar to that of bslo ($P > 0.05$) but different from that of mslo ($P < 0.001$) (Fig. 3a). These data seem to indicate that the presence of a valine residue in the S0–S1 loop ‘locks’ slo channels into (or shifts the channel population to) a state that is readily activatable by ethanol.

Protein phosphatase switches channel responses to ethanol

We used a sequence- and structure-based program for predicting eukaryotic protein phosphorylation sites (NetPhos 2.0 server) and found that slo Thr107, in contrast to valine, has a rather high probability (≥ 0.4) of being phosphorylated. This raised the possibility that different degrees of phosphorylation of Thr107 determine, or at least contribute to, the heterogeneity of bslo responses to alcohol.

Slo subunits contain several sites that may be phosphorylated by various cross-talking signaling molecules²²; this makes it difficult to determine, *a priori*, which phosphorylating signal should be probed so as to test the role of slo phosphorylation in modulating channel responses to ethanol. Thus, we first investigated whether nonselective dephosphorylation could lock slo channels containing Thr107 into an ethanol-activatable state. We treated I/O patches with alkaline phosphatase to dephosphorylate the bslo subunit (and nearby associated proteins; see Methods).

Alkaline phosphatase treatment increased channel activity to 192.6% of the values before alkaline phosphatase ($n = 10$), as reported with native BK channels in bovine tracheal myocytes³¹. These channels contain slo subunits identical in sequence to those used in our study. Thus, accessory β_1 -subunits, which are tightly associated with bslo in the native channel² but absent in our expression system¹¹, seem to be unnecessary for alkaline phosphatase to modulate bovine tracheal BK channels.

Before alkaline phosphatase treatment, the NP_o responses of bslo channels to ethanol were typically heterogeneous and could be grouped into potentiation (that is, 'activatable channel population'; $n = 5$) and refractoriness or inhibition (that is, 'nonactivatable channel population'; $n = 5$) (Fig. 4a). In this nonactivatable population, ethanol responses averaged $83.7 \pm 9.2\%$ of pre-ethanol controls. In sharp contrast, after alkaline phosphatase incubation of the same excised patches for 5 min, both activatable and nonactivatable bslo channels were activated by ethanol (Fig. 4b), with overall NP_o in the presence of ethanol averaging $242.3 \pm 22.8\%$ of that in the pre-ethanol controls ($P < 0.01$). This qualitative switch in the ethanol responses of bslo channels cannot be explained by ethanol modification of alkaline phosphatase activity, as ethanol was applied to the patch > 5 min after phosphatase was removed from the bath. Rather, the switch in ethanol responses of bslo channels by alkaline phosphatase is probably a result of dephosphorylation of a target in the membrane patch, perhaps the slo subunit itself.

Notably, after alkaline phosphatase treatment, ethanol activation of the previously nonactivatable population of channels was similar to its effect on the activatable population before the application of alkaline phosphatase ($P > 0.05$) (Fig. 4a). Furthermore, alkaline phosphatase did not modify ethanol responses in the activatable population of channels (Fig. 4a). Thus, channels characterized by an ethanol response that was unmodified by alkaline phosphatase seem to remain in an ethanol-activatable state before being exposed to the phosphatase.

In contrast to the bslo results, alkaline phosphatase did not modify bslo T107V ethanol responses; NP_o reached $223 \pm 24.6\%$ and $229.6 \pm 18.9\%$ of pre-ethanol controls ($P > 0.05$; $n = 7$) before and after dephosphorylating treatment. Therefore, alkaline phosphatase-mediated dephosphorylation probably modifies bslo channel responses to ethanol by targeting Thr107. The results from the alkaline phosphatase experiments on bslo and bslo T107V support the idea that the dephosphorylation of Thr107 locks the channel population into a state or states readily activatable by ethanol. Next, we explored which phosphorylation signaling molecule was involved in modulating ethanol action.

CaMKII modifies BK activity and its modulation by ethanol

The sequence KEETV in the bslo S0–S1 loop is similar to RQES/TV, a CaMKII conserved motif²⁸. Thus, we hypothesized that CaMKII phosphorylation before patch excision from the cell modifies slo Thr107 and, thus, channel responses to ethanol.

We first determined whether CaMKII could be detected in the intact cell under our recording conditions. Western analysis using polyclonal antibodies (Santa Cruz Biotechnology) identified an ~50-kDa band corresponding to CaMKII (J.L., A.M.D. and S. Tavalin, unpublished observations). This finding is consistent with a previous report identifying CaMKII in *Xenopus* oocytes³². Next, we used KN-93, a rather selective CaMKII inhibitor³³, to probe any involvement of the CaMKII phosphorylation of Thr107 in slo channel responses to ethanol.

After cells were incubated with KN-93 (20 μ M for 15 min), all bslo channels were activated by ethanol, with average NP_o increasing to $242.3 \pm 12.9\%$ of pre-ethanol controls ($n = 9$; Fig. 5). This response was markedly different from the heterogeneous pattern and from the average response evoked by ethanol in the absence of KN-93 ($P < 0.001$; $n = 16$). Furthermore, ethanol responses of bslo channels in the presence of KN-93 were similar to the responses evoked by ethanol in mslo and bslo T107V ($P > 0.05$; Fig. 3), both channels lacking the phosphorylatable Thr107 in the S0–S1 loop.

In contrast, KN-92, an analog of KN-93 that does not inhibit CaMKII, failed to shift the bslo channel population to a state that was readily activatable by ethanol ($n = 9$). Indeed, ethanol responses of bslo channels in KN-92 were similar to those in the absence of CaMKII inhibitor ($P > 0.05$; Fig. 5). Moreover, the ethanol responses of bslo channels in the presence of KN-92 were different from the ethanol responses of bslo channels in the presence of KN-93 (Fig. 5), and also from the responses of mslo and bslo T107V channels ($P < 0.01$).

In contrast to the results obtained with bslo, the ethanol responses of bslo T107V channels were similar before and after KN-93 treatment ($P > 0.05$) (Figs. 3b and 5). Together, these results strongly suggest that CaMKII modification of the ethanol responses of slo channels is a result of the phosphorylation of slo Thr107 by the kinase. Data obtained with selective inhibitors strongly suggest that endogenous basal CaMKII activity in the intact cell modulates ethanol responses of BK channels. We repeated these experiments with the different channel isoforms and mutants under a variety of treatments (Table 1).

To investigate, more directly, the role of CaMKII in modulating the ethanol responses of slo channels, we activated CaMKII *in vitro* with ATP + calmodulin and applied this phosphorylating complex (activated CaMKII + ATP + calmodulin) to I/O patches expressing bslo. After 5 min of incubation, we washed the patch in a complex-free bath solution for >5 min and then evaluated the action of ethanol on bslo channel currents. The application of CaMKII + ATP + calmodulin increased bslo channel currents to ~167% of those in the control ($n = 5$; Fig. 6a,b, top panels), whereas the application of ATP + calmodulin repeatedly failed to increase current ($n = 6$). In contrast to bslo data, CaMKII + ATP + calmodulin did not modify bslo T107V currents ($95.7 \pm 16.4\%$ of values before applying the phosphorylating complex; $n = 6$). Together these results indicate that CaMKII potentiates bslo channel currents by phosphorylating slo Thr107.

To further evaluate a direct role of CaMKII-mediated phosphorylation in the alcohol responses of BK channels, we next focused on the ethanol-activatable bslo channel population. Currents from this population (Fig. 6a) were typically inhibited by ethanol after CaMKII phosphorylation treatment when evaluated in the same I/O patch (Fig. 6b). This result (Fig. 6) was replicated in four other patches, with average responses before (Fig. 6c) and after (Fig.

6d) CaMKII treatment shown as $G/G_{\max}$ versus V Boltzmann plots³⁴. The data demonstrate that neither ethanol nor CaMKII modified the voltage needed to produce an e -fold change in $G/G_{\max}$ (given by k , the slope of the fit). CaMKII shifted the $V_{0.5}$ of the $G/G_{\max}$ versus V relationship to the left: $V_{0.5} = 122.8 \pm 8.3$ mV and 102.8 ± 6.8 mV in the absence and presence, respectively, of activated CaMKII ($P < 0.05$).

Ethanol substantially shifted the $V_{0.5}$ to the left before CaMKII treatment (Fig. 6c) and to the right after CaMKII treatment (Fig. 6d) (all k and $V_{0.5}$ values are detailed in Supplementary Note online). In brief, both CaMKII and ethanol modified bslo currents by producing a parallel shift in the $G/G_{\max}$ versus V relationship. These data suggest that both modulators modify channel steady-state activity without altering the number of charges mobilized across the electric field to gate the channel.

The results clearly demonstrate that the ethanol potentiation of bslo currents is switched to ethanol inhibition after CaMKII phosphorylating treatment (Fig. 6). This switch in ethanol responses is a mirror image of that caused by alkaline phosphatase (Fig. 4a) and KN-93 (Fig. 5). Furthermore, as previously found with alkaline phosphatase or KN-93 treatments (Figs. 4b and 5), CaMKII+ATP+calmodulin failed to modify the ethanol responses of bslo T107V channels, which reached $104.8 \pm 12.8\%$ of controls ($n = 6$). Together these data suggest that it was the phosphorylation status of Thr107, secondary to CaMKII action, that determined the alcohol responses of slo channels.

CaMKII works as a molecular dimmer switch

The results seem to indicate that ethanol inhibits slo channels containing phosphorylated Thr107 while robustly activating channels containing dephosphorylated Thr107. Because BK channels are tetramers, the presence or absence of phosphate in different subunits could influence ethanol action, producing intermediate responses between robust potentiation and inhibition. Alternatively, the channel might respond to ethanol with a switch from robust activation to inhibition when a critical number of subunits is phosphorylated. To distinguish between these possibilities, we combined site-directed mutagenesis of Thr107 (to valine) with the mutation Y315V. The latter mutation in the extracellular vestibule of the channel (Fig. 1a) introduced resistance to external block by tetraethylammonium (TEA) in the assembled tetramer. Thus, by injecting oocytes with different molar ratios of bslo to bslo T107V Y315V (Methods), we determined the channel stoichiometry from measurements of unitary current amplitude in 2 mM external TEA, as previously described³⁴. After establishing a correspondence between channel stoichiometry and current amplitude (Supplementary Figure 1 online) and taking advantage of channel open probability (P_o) determinations from all-points amplitude histograms¹⁵, we evaluated the action of ethanol on channel P_o from tetramers made of different subunit combinations.

Channels constructed with different combinations of bslo and bslo T107V Y315V were treated with CaMKII + ATP + calmodulin (see above), and unitary currents were evoked before (Fig. 7a, left traces) and after (Fig. 7a, right traces) ethanol exposure. As expected, these traces showed a progressive reduction in unitary current amplitude in the presence of TEA as the number of bslo T107V Y315V subunits was decreased from four (top row) to one (fourth row). In all patches, CaMKII failed to modify TEA action (data not shown).

From patches containing a single channel of a given subunit combination, we could determine P_o values, following CaMKII treatment, before and after ethanol application. This condition (that is, $N = 1$) was very difficult to obtain owing to the well-known clustering of slo channels in the cell membrane^{11,15}. In four of four patches, the data obtained at constant transmembrane voltage (60 mV) and $[Ca^{2+}]_i$ (0.3 μ M) indicated that the incremental phosphorylation, by CaMKII, of Thr107 in the bslo tetramer progressively increased basal P_o : the P_o values were

0.010 ± 0.007, 0.023 ± 0.004, 0.058 ± 0.006, 0.108 ± 0.005 and 0.218 ± 0.006 (mean ± s.e.m.; $n = 4$) for tetramers having zero, one, two, three or four phosphorylated Thr107 subunits, respectively (Fig. 7a, left traces). This increase in channel steady-state activity is likely a primary contributor to the increase in macroscopic current caused by CaMKII treatment (Fig. 6a versus 6b, top traces).

Unitary current records showed the characteristic ethanol inhibition of channel activity after CaMKII treatment (Fig. 7a, bottom traces), with average values reaching $72.4 \pm 8.4\%$ of control (ethanol responses from bslo channels were evaluated in the absence of TEA because this agent totally blocked bslo currents, as has been reported with mslo channels³⁴). This ethanol decrease in P_o probably explains the ethanol inhibition of macroscopic current (Fig. 6). Alcohol inhibition of channel activity was lost when only one bslo T107V Y315V subunit was present in the tetramer; after CaMKII treatment, P_o in the presence of ethanol reached ~130% that of controls (Fig. 7a, fourth traces from top, and Fig. 7b, second column). Thus, ethanol inhibition of slo channels requires that all subunits in the tetramer be phosphorylated at Thr107. Our data indicate that CaMKII-mediated phosphorylation of this residue works as a molecular switch that shifts slo channel responses to ethanol from mild activation to inhibition (Fig. 7a,b).

The robust potentiation by ethanol of homotetramers containing un-phosphorylatable residues (valine) in the S0–S1 loop gradually decreased as bslo T107V Y315V subunits were progressively replaced by slo subunits with phosphorylated Thr107 (Fig. 7a,b). Average values for ethanol potentiation were $262.1 \pm 16.1\%$, $200.1 \pm 16.4\%$, $157.3 \pm 10.8\%$ and $120.1 \pm 7.9\%$ of pre-ethanol controls for tetramers having zero, one, two and three phosphorylated T107 subunits, respectively. Notably, the rate at which the ethanol potentiation of bslo channels changed with varying stoichiometry was constant (Fig. 7b), which suggested that increasing the number of dephosphorylated subunits did not introduce cooperativity in ethanol-induced channel activation. In conclusion, CaMKII phosphorylation of Thr107 works as a molecular dimmer switch, progressively diminishing the activating effect of alcohol and finally switching it to inhibition.

Mutations to negatively charged residues, such as aspartate and glutamate, have been used to imitate a phosphorylated residue in proteins. Owing to steric and intrinsic activity differences, however, these mutations do not always produce a functional correlate in the protein that exactly mimics the effects of a phosphorylated residue^{35,36}.

We mutated Thr107 to a phosphomimetic glutamate and studied ethanol action on bslo T107E channels. Glutamate was chosen over aspartate because it is widely accepted that the extra methylene group of glutamate provides a chain length that more closely resembles the side chain of a phosphorylated threonine.

In response to ethanol, the NP_o of bslo T107E channels reached $132.4 \pm 6.1\%$ of controls ($n = 11$). As expected for a phosphomimetic mutation, this response was different from that of bslo T107V in the same batch of oocytes ($247.8 \pm 17.7\%$ of controls; $P < 0.001$; Fig. 7c). Although not identical to the ethanol responses of bslo in the presence of CaMKII, the ethanol responses of bslo T107E homotetramers (Fig. 7c, middle column) were statistically indistinguishable ($P > 0.05$) from those evoked in heterotetramers containing two bslo T107V Y315V subunits (Fig. 7b, third column). Thus, slo tetramers having a different subunit composition but the same number of negative charges in S0–S1 (that is, four) respond similarly to ethanol. In brief, the results obtained with bslo T107E partially mimicked the ethanol responses evoked in CaMKII-treated bslo channels, which is consistent with the idea that glutamate partially mimics a phosphorylated threonine. The results obtained with phosphomimetic bslo T107E support our conclusion that the phosphorylation of Thr107 progressively diminishes ethanol potentiation of BK channels.

DISCUSSION

A slo point mutation controls BK channel ethanol responses

Acute ethanol exposure modifies the activity of several ligand-gated ion channels, with drug action being altered by changes in one or several amino acids in the channel protein^{37–41}. In contrast, with the exception of G protein-coupled inwardly rectifying K⁺ channel (GIRK2)⁴² and Shaw2 (ref. 29), most members of the K⁺ ion channel superfamily are resistant to clinically relevant concentrations of ethanol³⁰. Slo channels possess the S0 segment and S0–S1 loop, which are absent in all other voltage-gated K⁺ channels² (Fig. 1a). Notably, we identified a critical residue in the S0–S1 loop (Thr107) that controls mammalian BK channel responses to ethanol. This residue represents a primary site that links ethanol-induced conformational changes in the channel protein to channel gating.

Our results can be interpreted in two ways. First, ethanol binds to the S0–S1 loop and, eventually, modifies channel gating. If so, the putative binding site should have very lax structural constraints. Our studies show that for ethanol to robustly activate slo channels, it is not important whether the ‘binding site’ contains valine (as in mslo channels), alanine (as in hslo channels) or dephosphorylated threonine (as in the bslo activatable population). Thus, amino acids differing in polarity, chain length or both should all bind ethanol. Second, ethanol does not bind to the S0–S1 loop but to a different site in the slo subunit, its lipid microenvironment or both. If so, mutations in S0–S1 that affect drug action should modify slo conformation and, thus, alter the conformational changes that occur upon ethanol binding to a region other than the S0–S1 loop.

A major change in the secondary structure of S0–S1 by a point mutation is supported by modeling data, which show that mutating Thr107 to valine causes a shift from a predominantly coiled structure to a predominantly α -helix structure (Fig. 1b). However, bslo treated with alkaline phosphatase (which shifts the Thr107 to its dephosphorylated state) and bslo T107V channels responded identically to ethanol (Fig. 4), which suggests that the secondary structure of the S0–S1 loop *per se* is not a major determinant of ethanol responses. Rather, ethanol action probably involves additional structures outside the S0–S1 loop. Previous data with bslo-mslo chimeras¹² and the present data showing quantitative differences in ethanol responses between mslo and bslo T107V channels (Fig. 3) seem to indicate that the slo tail domain contains one or more modulatory sites that additionally regulate the responses of the slo channel to ethanol.

A study in *C. elegans* has identified two residues in slo that modify channel responses to ethanol and condition alcohol-induced motor incoordination⁹. One residue (cslo Ile1001), located in the S10 segment of the slo tail domain, is not conserved between *C. elegans* and mammalian species. The second (cslo Lys350), located in S6, is conserved among species, including mice, rodents, bovine and humans. Notably, *C. elegans* slo channels that are activated by ethanol contain a nonphosphorylatable residue (glutamic acid) in the S0–S1 loop, as found in our study (bslo T107V). The bslo isoform we used contains the S6 lysine residue, but a phosphorylated Thr107 renders the channels resistant to ethanol activation (Figs. 3 and 5–7). Thus, our data suggest that in mammalian (or, at least, in the bovine) species, the role of the S6 lysine in ensuring slo channel activation by ethanol may be overridden by CaMKII phosphorylation of Thr107.

CaMKII modifies P_0 and gradually switches ethanol action

Different kinases modulate BK channel activity^{18,22}, and phosphorylation sites for protein kinases A, G and C (PKA, PKG and PKC) have been identified in the slo tail^{2,3}. We now report a distinct region (the S0–S1 loop) that contains a critical residue targeted by CamKII phosphorylation, which leads to eventual modification of slo channel activity. We cannot

totally rule out the possibility that CaMKII activates slo channels by phosphorylating an accessory protein(s) tightly associated to slo. Our data, however, demonstrate that bslo T107V channels are insensitive to CaMKII or alkaline phosphatase modulation. Thus, it is very likely that CaMKII activates bslo channels by directly phosphorylating Thr107 in the S0–S1 loop.

Consistent with the importance of Thr107 in CaMKII modulation of channel activity, a CaMKII inhibitory peptide fails to modulate mslo channels⁴³, which have an alanine instead of a threonine at position 107. Mslo, however, is modulated by ATP, a process that might involve CaMKII⁴³. Our data demonstrate that ATP does not directly modulate bslo channels, as the application of calmodulin and ATP did not modify bslo currents in I/O macropatches. Thus, it seems that CaMKII phosphorylation of bslo Thr107 does not occur constitutively in the excised patch. This result is in contrast to other kinases, which seem to remain active and modulate BK channels in excised cell-free patches²².

Bslo channels that have been targeted by CaMKII have higher basal activity and are inhibited by ethanol. Furthermore, progressive CaMKII phosphorylation of Thr107 in the channel tetramer gradually increases basal P_o and diminishes ethanol activation. Thus, it could be argued that in the fully phosphorylated tetramer, the channel reaches a state of very high P_o that cannot be further increased (but inhibited) by ethanol. Our studies, however, were conducted at low P_o , as demonstrated by the linearity of the $\ln(NP_o)$ -voltage relationship (see also P_o values in Fig. 7a); this rules out the possibility that the channel reached near maximal activation before ethanol application.

Both ethanol and CaMKII phosphorylation of Thr107 increase slo currents without modifying channel unitary conductance. Modulation of channel function seems restricted to increases in steady-state activity, which occurs without modifying the voltage dependence of channel gating. It is conceivable that the action of ethanol on channel gating might result from alcohol mimicking a phosphorylation process on slo Thr107. The modification of channel function by ethanol, however, cannot be secondary to ethanol activation of CaMKII itself, because the alcohol was added to the patch long after the enzyme was washed out of the system. In addition, because the action of ethanol on channel function was evaluated several minutes after patch excision in the absence of nucleotides, we can rule out the need for the continuous presence of other cytosolic (de)phosphorylating agents in ethanol action.

Functional implications

BK channels critically determine vascular smooth muscle tone⁴⁴ and control fast action potential after-hyperpolarizations, setting the firing properties of neurons⁴. Moreover, activation of this channel type leads to depressed excitability in a variety of neurons and neuroendocrine cells⁶. As found for BK channels, CaMKII activity controls both vascular smooth muscle tone²⁷ and neuronal spike repolarization²⁶. Thus, our findings provide a new potential mechanism—namely, the modulation of BK channel activity by CaMKII targeting of the slo S0–S1 loop—by which changes in CaMKII activity may alter vascular tone, neuronal excitability or both. Given the fact that all neuronal slo channels cloned so far, from *C. elegans* to human, contain nonphosphorylatable residues in positions equivalent to bslo Thr107, this single molecular determinant may help explain why neuronal BK channels are almost universally activated by ethanol.

The final response of a native BK channel to alcohol, however, results from a combination of factors. The expected activation in slo containing a nonphosphorylatable residue in the S0–S1 loop may be modulated by accessory proteins. For example, ethanol potentiation of BK channels in cell bodies of nucleus accumbens neurons in the rat has been related primarily to the overexpression of BK β_4 -subunits⁴⁵. In contrast, in cell-free patches of rat cerebrovascular myocytes, ethanol produces a transient channel activation (< 2 min), consistent with the

presence of slo isoforms containing nonphosphorylatable residues in the S0–S1 loop (AY330293 and AY330294). Activation is followed by inhibition, probably reflecting channel desensitization¹². Superimposed on this dual drug action on the BK channel itself, ethanol robustly inhibits Ca²⁺ sparks, which indirectly leads to BK channel inhibition. This indirect action seems to prevail over the initial channel activation, as inhibition of cerebral artery BK currents occurs as soon as ethanol is applied and is sustained as long as the drug is present, leading to cerebral artery constriction¹².

Finally, the dimmer switch mechanism by which the CaMKII phosphorylation of slo Thr107 modulates the ethanol responses of BK channels might represent a form of acute tolerance for alcohol in mammals. Notably, CaMKII activity in astrocytes is increased following protracted ethanol exposure⁴⁶. Thus, CaMKII might represent a signaling molecule operating in negative feedback mechanisms on BK channel responses to alcohol. The proposed dimmer switch mechanism, however, will be relevant in BK channels containing S0–S1 sequences similar to that of bslo. Bslo is found in bovine aortic smooth muscle and brain tissue (AY862880). In this particular species, the phosphorylation of bslo might represent a protective mechanism in response to high blood ethanol levels following the production of ethanol in the rumen (following food fermentation) or the absorption of ethanol added to food⁴⁷.

In contrast, most slo isoforms from other species, including humans, lack phosphorylatable residues in the S0–S1 loop. Differential ethanol responses among slo channels, however, do not necessarily have to be interpreted as species specific. We have recently identified slo subunits in the bovine brain that have a short S0–S1 loop and contain nonphosphorylatable residues (AY862879), as do their human and rodent counterparts. Conversely, we cannot rule out the possibility that slo isoforms containing phosphorylatable residues in the S0–S1 loop will be identified in species other than bovine, raising the possibility that the mechanism described in our study could represent a rather generalized way to control BK channel responses to ethanol.

In conclusion, we have pinpointed a previously unidentified single residue in the slo subunit and a defined post-translational mechanism that determine BK channel activity and its response to alcohol. The presence of nonphosphorylatable amino acids in the slo S0–S1 loop in the vast majority of neuronal BK channels across species may provide a molecular basis for the widely reported activation of this channel type in neurons by intoxicating concentrations of alcohol.

METHODS

Mutagenesis, cRNA synthesis and protein expression

Mslo (mbr5) and bslo cDNAs were a gift from L. Salkoff and E. Moczydlowski. cDNAs inserted into pBluescript vector were cut with *Cla*I and *Not*I and reinserted into *pBscMXT* vector for expression in *Xenopus* oocytes. *Slo* mutants were constructed using Quickchange (Stratagene) and their sequences were confirmed at the University of Tennessee Molecular Research Center. *Mslo* and *bslo* cDNAs were linearized with *Sal*I and *Xba*I and transcribed *in vitro* using T3 polymerase.

Following their removal and defolliculation¹⁵, oocytes were transferred into ND-96 saline¹⁵. cRNA (0.1–1 µg µl⁻¹; 19–37 nl) was injected using an automated microinjector (Drummond). Tetramers with different bslo and bslo T107V Y315V stoichiometry were obtained by injecting oocytes with bslo and bslo T107V Y315V cRNAs at various molar ratios (from 1:1 to 1:50). Animal research protocols were approved by the Animal Care and Use Committee at the University of Tennessee Health Science Center.

Electrophysiological recordings

Before recording, the oocyte vitelline layer was removed as previously described¹⁵. Oocytes were placed into gentamicin-free, ND-96 saline¹⁵ for 15 min. Single channel recordings were conducted 48–72 h after cRNA injection from I/O patches using patch-clamp techniques¹⁵. The bath solution contained 130 mM potassium gluconate, 3.84 mM CaCl₂, 1 mM MgCl₂, 5 mM EGTA and 15 mM HEPES at pH 7.35; free [Ca²⁺] ≈ 300 nM. The electrode solution contained 130 mM potassium gluconate, 5.22 mM CaCl₂, 2.28 mM MgCl₂, 5 mM EGTA, 1.6 mM HEDTA and 15 mM HEPES at pH 7.35; free [Ca²⁺] = 11 ± 0.6 μM. Free [Ca²⁺] was calculated using Max Chelator Sliders (C. Patton) and measured with Ca²⁺ electrodes (Corning).

Patch pipettes were pulled from glass capillaries (Drummond); the electrode shank was coated with Sylgard 184 (Dow Corning). Before recordings, pipette tips were fire-polished on a microforge (WPI), yielding tip resistances of 5–10 MΩ when filled with electrode solution. An Ag/AgCl electrode was used as ground electrode. After excision from the oocyte, the membrane patch was exposed to bath solution containing the desired concentrations of ethanol and Ca²⁺ flowing from an automated perfusion system (ALA Scientific Instruments). Deionized 100% pure ethanol (American Bioanalytical) was diluted in bath solution immediately before experiments. Perfusion with urea isosmotically replacing ethanol was used as control perfusion. Experiments were carried out at 20 °C.

Currents were recorded using an EPC8 amplifier (List), low-pass filtered at 1 kHz and sampled at 10 kHz. Data acquisition and analysis were performed using pClamp 8 (Axon). The product of number of channels in the patch (*N*) and the open channel probability (*P*_o) was used as an index of channel steady-state activity¹⁵. Voltages given are the potential at the intracellular side of the patch.

To determine channel modulation by CaMKII phosphorylation of Thr107, macroscopic currents were evoked from a holding potential of –80 mV with 200-ms long depolarizing voltage steps (10-mV increments) from –40 mV to 160 mV in I/O macropatches. Steady-state current amplitude was determined 175–200 ms after the beginning of the voltage step. *G*/*G*_{max} versus *V* plots were fitted to a Boltzmann function: $G(V) = G_{\max} / (1 + e^{-(V - V_{0.5})/k})$. Bath and electrode solutions had compositions identical to those described for single channel recordings.

Alkaline phosphatase treatment

Membrane patches expressing slo were excised from the cell in symmetric conditions at 0 mV. After 5 min of recording the channel NP_o, the intracellular side of the I/O patch was placed near the micropipette mouth of a perfusion system from which bath solution containing either 100 mM ethanol or the isosmotic control was applied to the patch for ≤2 min, NP_o being determined under alternative ethanol-containing or control perfusion. Alkaline phosphatase was added to the bath (final alkaline phosphatase concentration 33 IU ml^{–1}) 3 min after recovery from the first ethanol application. After 5 min of alkaline phosphatase application, the bath solution was replaced with enzyme-free solution, and channel NP_o (alkaline phosphatase-treated control) was determined. Then ethanol action was evaluated again and compared to alkaline phosphatase control values.

KN-93 and KN-92 treatments

After devitellinization, oocytes were placed into ND-96 saline containing 20 μM KN-93 (Calbiochem), a selective CaMKII inhibitor³³, for 15 min. Then NP_o was recorded in the absence and presence of ethanol. As control, we used KN-92 (Calbiochem), a biologically inert analog of KN-93.

CaM kinase II treatment

CaMKII (800 IU) (New England Biolabs) was diluted in 40 μ l of reaction buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 2 mM DTT, 0.1 mM Na₂EDTA supplemented with 0.1 ATP, 0.0012 mM calmodulin and 2 mM CaCl₂, for 10 min at 30 °C. Activated CaMKII (20,000 IU ml⁻¹) was put on ice for a few minutes, ready to be applied to I/O patches.

After patch excision, we evaluated channel NP_o in ethanol versus the control. Only I/O patches containing bslo channels that were activated by ethanol were exposed to the CaMKII phosphorylating complex: 70 μ l of 10 $\times$ CaMKII reaction buffer and 1,000 IU activated CaMKII were added to the bath solution and incubated for 5 min. Then the whole bath solution was replaced with CaMKII-free solution and, after an interval of >5 min, channel NP_o was recorded before (isosmotic control) and after exposing the cytosolic side of the patch to ethanol.

To address the role of slo stoichiometry in CaMKII modulation of ethanol action, the activity of channels containing bslo T107V Y315V was determined in the presence of external TEA (2 mM). To better resolve the slo currents partially blocked by TEA from endogenous, mechanogated cation currents, these studies were conducted in 60 μ M GdCl₃³⁴.

Data analysis

Data are expressed as mean $\pm$ s.e.m.; n = number of patches. Data were analyzed using pStat v.6 (Axon) and Instat v.3.05 (GraphPad). Statistical analysis was performed using one-way ANOVA followed by Bonferroni's test. Data were plotted and fitted using Origin v.6.1 (Originlab).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank S. Bahouth, S. Tavalin, J. Jaggar and D. Armbruster for critically reading the manuscript, and K. Malik for helpful discussion. This work was supported by the US National Institutes of Health (grants AA11560 and HL77424 to A.M.D.).

References

1. Kaczorowski GJ, Knaus HG, Leonard RJ, McManus OB, Garcia ML. High-conductance calcium-activated potassium channels; structure, pharmacology, and function. *J Bioenerg Biomembr* 1996;28:255–267. [PubMed: 8807400]
2. Toro L, Wallner M, Meera P, Tanaka Y. Maxi-K_{Ca}, a unique member of the voltage-gated K⁺ channel superfamily. *News Physiol Sci* 1998;13:112–117. [PubMed: 11390773]
3. Tian L, et al. Alternative splicing switches potassium channel sensitivity to protein phosphorylation. *J Biol Chem* 2001;276:7717–7720. [PubMed: 11244090]
4. Sah P, Faber ES. Channels underlying neuronal calcium-activated potassium currents. *Prog Neurobiol* 2002;66:345–353. [PubMed: 12015199]
5. Krnjevic, K. Excitable membranes. In: Fink, BR., editor. *Cellular Biology and Toxicity of Anesthetics*. Williams and Wilkins; Baltimore: 1972. p. 3-9.
6. Crowder CM. Ethanol targets: a BK channel cocktail in *C. elegans*. *Trends Neurosci* 2004;27:579–582. [PubMed: 15374666]
7. Gruss M, et al. Ethanol reduces excitability in a subgroup of primary sensory neurons by activation of BK_{Ca} channels. *Eur J Neurosci* 2001;14:1246–1256. [PubMed: 11703454]

8. Knott TK, Dopico AM, Dayanithi G, Lemos J, Treistman SN. Integrated channel plasticity contributes to alcohol tolerance in neurohypophysial terminals. *Mol Pharmacol* 2002;62:135–142. [PubMed: 12065764]
9. Davies AG, et al. A central role of the BK potassium channel in behavioral responses to ethanol in *C. elegans*. *Cell* 2003;115:655–666. [PubMed: 14675531]
10. Walters FS, Covarrubias M, Ellingson JS. Potent inhibition of the aortic smooth muscle maxi-K channel by clinical doses of ethanol. *Am J Physiol Cell Physiol* 2000;279:C1107–C1115. [PubMed: 11003591]
11. Dopico AM. Ethanol sensitivity of BK_{Ca} channels cloned from arterial smooth muscle does not require the presence of the beta 1 subunit. *Am J Physiol Cell Physiol* 2003;284:C1468–C1480. [PubMed: 12570985]
12. Liu P, Xi Q, Ahmed A, Jaggar JH, Dopico AM. Essential role for smooth muscle BK channels in alcohol-induced cerebrovascular constriction. *Proc Natl Acad Sci USA* 2004;101:18217–18222. [PubMed: 15604147]
13. Zakhari S. Alcohol and the cardiovascular system: molecular mechanisms for beneficial and harmful action. *Alcohol Health Res World* 1997;21:21–29. [PubMed: 15706760]
14. Dopico AM, Widmer H, Wang G, Lemos JR, Treistman SN. Rat supraoptic magnocellular neurones show distinct large conductance, Ca²⁺-activated K⁺ channel subtypes in cell bodies versus nerve endings. *J Physiol (Lond)* 1999;519:101–104. [PubMed: 10432342]
15. Dopico AM, Anantharam V, Treistman SN. Ethanol increases the activity of Ca⁺⁺-dependent K⁺ (*mslo*) channels: functional interaction with cytosolic Ca⁺⁺. *J Pharmacol Exp Ther* 1998;284:258–268. [PubMed: 9435186]
16. Liu P, Liu J, Huang W, Li MD, Dopico AM. Distinct regions of the slo subunit determine differential BK_{Ca} channel responses to ethanol. *Alcohol Clin Exp Res* 2003;27:1640–1644. [PubMed: 14574235]
17. Crowley JJ, Treistman SN, Dopico AM. Cholesterol antagonizes ethanol potentiation of human brain BK_{Ca} channels reconstituted into phospholipid bilayers. *Mol Pharmacol* 2003;64:365–372. [PubMed: 12869641]
18. Weiger TM, Hermann A, Levitan IB. Modulation of calcium-activated potassium channels. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 2002;188:79–87. [PubMed: 11919690]
19. Solem M, McMahon T, Messing RO. Protein kinase A regulates inhibition of N- and P/Q-type calcium channels by ethanol in PC12 cells. *J Pharmacol Exp Ther* 1997;282:1487–1495. [PubMed: 9316863]
20. Valenzuela CF, Cardoso RA, Lickteig R, Browning MD, Nixon KM. Acute effects of ethanol on recombinant kainate receptors: lack of role of protein phosphorylation. *Alcohol Clin Exp Res* 1998;22:1292–1299. [PubMed: 9756045]
21. Jakab M, Weiger TM, Hermann A. Ethanol activates maxi Ca²⁺-activated K⁺ channels of clonal pituitary (GH3) cells. *J Membr Biol* 1997;157:237–245. [PubMed: 9178611]
22. Schubert R, Nelson MT. Protein kinases: tuners of the BKCa channel in smooth muscle. *Trends Pharmacol Sci* 2001;22:505–512. [PubMed: 11583807]
23. Erondy N, Kennedy M. Regional distribution of type II Ca²⁺/calmodulin-dependent protein kinase in rat brain. *J Neurosci* 1985;5:3270–3277. [PubMed: 4078628]
24. Greenwood IA, Leblanc N, Ledoux J. Differential regulation of Ca²⁺-activated Cl[−] currents in rabbit arterial and portal vein smooth muscle cells by Ca²⁺-calmodulin-dependent kinase. *J Physiol (Lond)* 2001;534:395–408. [PubMed: 11454959]
25. Fink CC, Meyer T. Molecular mechanisms of CaMKII activation in neuronal plasticity. *Curr Opin Neurobiol* 2002;12:293–299. [PubMed: 12049936]
26. Eshete F, Fields R. Spike frequency decoding and autonomous activation of Ca²⁺-calmodulin-dependent protein kinase II in dorsal root ganglion neurons. *J Neurosci* 2001;21:6694–6705. [PubMed: 11517259]
27. Kim I, et al. Ca²⁺-calmodulin-dependent protein kinase II-dependent activation of contractility in ferret aorta. *J Physiol (Lond)* 2000;526:367–374. [PubMed: 10896725]
28. Pearson RB, Kemp BE. Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations. *Methods Enzymol* 1991;200:62–81. [PubMed: 1956339]

29. Covarrubias M, Vyas TB, Escobar L, Wei A. Alcohols inhibit a cloned potassium channel at a discrete saturable site. Insights into the molecular basis of general anesthesia. *J Biol Chem* 1995;270:19408–19416. [PubMed: 7642622]
30. Harris RA. Ethanol actions on multiple ion channels: which are important? *Alcohol Clin Exp Res* 1999;23:1563–1570. [PubMed: 10549986]
31. Zhou XB, Ruth P, Schlossmann J, Hofmann F, Korth M. Protein phosphatase 2A is essential for the activation of Ca^{2+} -activated K^{+} currents by cGMP-dependent protein kinase in tracheal smooth muscle and Chinese hamster ovary cells. *J Biol Chem* 1996;271:19760–19767. [PubMed: 8702682]
32. Stevens I, Rondelez E, Merlevede W, Goris J. Cloning and differential expression of new calcium, calmodulin-dependent protein kinase II isoforms in *Xenopus laevis* oocytes and several adult tissues. *J Biochem* 2001;129:551–560. [PubMed: 11275554]
33. Mamiya N, et al. Inhibition of acid secretion in gastric parietal cells by the Ca^{2+} /calmodulin-dependent protein kinase II inhibitor KN-93. *Biochem Biophys Res Commun* 1993;195:608–615. [PubMed: 7690557]
34. Niu X, Magleby KL. Stepwise contribution of each subunit to the cooperative activation of BK channels by Ca^{2+} . *Proc Natl Acad Sci USA* 2002;99:11441–11446. [PubMed: 12161564]
35. Derkach V, Barria A, Soderling TR. Ca^{2+} /calmodulin-kinase II enhances channel conductance of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors. *Proc Natl Acad Sci USA* 1999;96:3269–3274. [PubMed: 10077673]
36. Mansour SJ, Candia JM, Matsuura JE, Manning MC, Ahn NG. Interdependent domains controlling the enzymatic activity of mitogen-activated protein kinase kinase 1. *Biochemistry* 1996;35:15529–15536. [PubMed: 8952507]
37. Mihic SJ, et al. Sites of alcohol and volatile anaesthetic action on GABA(A) and glycine receptors. *Nature* 1997;389:385–389. [PubMed: 9311780]
38. Sessoms-Sikes JS, Hamilton ME, Liu LX, Lovinger DM, Machu TK. A mutation in transmembrane domain II of the 5-hydroxytryptamine (3A) receptor stabilizes channel opening and alters alcohol modulatory actions. *J Pharmacol Exp Ther* 2003;306:595–604. [PubMed: 12730353]
39. Borghese CM, Ali DN, Bleck V, Harris RA. Acetylcholine and alcohol sensitivity of neuronal nicotinic acetylcholine receptors: mutations in transmembrane domains. *Alcohol Clin Exp Res* 2002;26:1764–1772. [PubMed: 12500999]
40. Ren H, Honse Y, Peoples RW. A site of alcohol action in the fourth membrane-associated domain of the N-methyl-D-aspartate receptor. *J Biol Chem* 2003;278:48815–48820. [PubMed: 14506267]
41. Mascia MP, Mihic SJ, Valenzuela CF, Schofield PR, Harris RA. A single amino acid determines differences in ethanol actions on strychnine-sensitive glycine receptors. *Mol Pharmacol* 1996;50:402–406. [PubMed: 8700149]
42. Lewohl JM, et al. G protein-coupled inwardly rectifying potassium channels are targets of alcohol action. *Nat Neurosci* 1999;2:1084–1090. [PubMed: 10570485]
43. Müller M, Madan D, Levitan IB. State-dependent modulation of mSlo, a cloned calcium-dependent potassium channel. *Neuropharmacology* 1996;35:877–886. [PubMed: 8938718]
44. Brayden JE, Nelson MT. Regulation of arterial tone by activation of calcium-dependent potassium channels. *Science* 1992;256:532–535. [PubMed: 1373909]
45. Martin G, et al. Somatic localization of a specific large-conductance calcium-activated potassium channel subtype controls compartmentalized ethanol sensitivity in the nucleus accumbens. *J Neurosci* 2004;24:6563–6572. [PubMed: 15269268]
46. Smith TL, Navratilova E. Increased calcium/calmodulin protein kinase activity in astrocytes chronically exposed to ethanol: influences on glutamate transport. *Neurosci Lett* 1999;269:145–148. [PubMed: 10454153]
47. Randby AT, Selmer-Olsen I, Baevre L. Effect of ethanol in feed on milk flavor and chemical composition. *J Dairy Sci* 1999;82:420–428. [PubMed: 10068963]

Figure 1.

BK channels are made of pore-forming α -(slo) and modulatory β -subunits. **(a)** Depiction of a BK channel heterodimer showing its α - and β -subunits. Four slo subunits are thought to assemble together to make a functional BK channel. The diagram indicates the slo pore region (P), the domain that regulates the conductance of K^+ (RCK domain), the Ca^{2+} bowl, and the core and tail domains, with the last two being joined by the linker. In the S0–S1 loop, the primary sequence alignment of key amino acids from bslo, mslo and hslo is shown; nonconserved residues are underlined. In the P region, the point mutation that introduces resistance to external TEA block is indicated. Accession numbers are given in parentheses. **(b)** Putative secondary structure of the S0–S1 loop and adjacent structures from mslo (left) and bslo (right). Columns represent the probability of finding a given secondary structure, as predicted by the PSIPRED server. C, coil; H, α -helix.

Figure 2.

Ethanol at clinically relevant concentrations increases bslo T107V channel activity (NP_o) after expression of these slo subunits in *Xenopus* oocytes. (**a–c**) Traces showing single channel recordings from the same I/O membrane patch obtained before (**a**), during (**b**) and 5 min after (**c**) exposure of the cytosolic side of the patch to 100 mM ethanol. Channel openings are shown as upward deflections; arrows indicate the baseline level. Channel NP_o was obtained from all-points amplitude histograms¹⁵, constructed from 60 s of continuous recording under each condition. The composition of the high $[K^+]$ solution facing both intracellular and extracellular sides of the patch is given in Methods. The membrane potential was set to 40 mV and the nominal free intracellular Ca^{2+} concentration was 300 nM.

Figure 3.

The valine-to-threonine mutation in the slo S0–S1 loop significantly modifies BK channel responses to acute ethanol exposure. **(a)** Average changes in channel NP_0 in response to ethanol are shown as mean $\pm$ s.e.m. where n (number of patches or oocytes) is shown in parentheses at the bottom of each bar graph. In the presence of ethanol, NP_0 ratios reached $106.1 \pm 15.6\%$ (wild-type bslo), $123.6 \pm 13.4\%$ (mslo V86T), $247.8 \pm 15.3\%$ (bslo T107V) and $196.3 \pm 16.7\%$ (wild-type mslo). * $P < 0.001$ and # $P < 0.01$ in comparison to wild-type bslo; [¶] $P < 0.001$ and [‡] $P < 0.05$ in comparison to mslo V86T. The statistical difference between individual means was determined by one-way ANOVA followed by Bonferroni's multiple-comparison test. **(b)** Acute exposure of the cytosolic side of I/O patches to 100 mM ethanol evoked varied responses in wild-type bslo and mslo V86T: activation (filled circles) or refractoriness/inhibition (hollow circles). In contrast, it consistently activated wild-type mslo and bslo T107V channels following subunit expression in the same batches of oocytes. The scatter graph depicts ratios of NP_0 values obtained in the presence and absence of ethanol ($\times 100$); each data point represents an individual patch or oocyte. In both **a** and **b**, the dashed line indicates the point at which NP_0 is unchanged by ethanol.

Figure 4. *In vitro* treatment with alkaline phosphatase shifts wild-type bslo channels into a state that is readily activatable by ethanol, but does not modify bslo T107V channel responses to alcohol (a) Before alkaline phosphatase (AP) treatment, acute exposure of the cytosolic side of I/O patches to 100 mM ethanol evoked varied responses in wild-type bslo: robust activation (filled circles) and refractoriness/inhibition (hollow circles). In contrast, after AP application (33 IU ml^{-1} for 5 min) to the same patches, ethanol potentiated all wild-type bslo channels. (b) Ethanol responses of bslo T107V channels were identical before and after AP is applied to the same I/O membrane patches. In both **a** and **b**, the scatter graphs depict ratios of NP_o values ($\times 100$) obtained in the presence and absence of ethanol; each data point represents an individual patch or oocyte. Ethanol responses in a given patch before and after AP are connected by a dotted line. The dashed line indicates the point at which NP_o is unchanged by alcohol.

Figure 5.

In vitro treatment with KN-93, a selective CaMKII inhibitor, shifted wild-type bslo channels into a state that is readily activatable by ethanol, but it does not modify bslo T107V channel responses to alcohol. Acute exposure of the cytosolic side of I/O patches to 100 mM ethanol evoked varied responses from wild-type bslo channels: activation (filled circles) and refractoriness/inhibition (open circles). In sharp contrast, after KN-93 treatment (20 μ M for 15 min), all wild-type bslo channels were activated by ethanol. Consistently, KN-92, an inactive analog of KN-93, failed to modify wild-type bslo channel responses. In contrast to wild-type bslo results, KN-93 treatment did not modify bslo T107V channel responses to ethanol when this subunit was expressed in the same batches of oocytes (compare the third column of data with the third column in Fig. 3b). The scatter graph depicts ratios of NP_o values ($\times 100$) obtained in the presence and absence of ethanol; each data point represents an individual patch or oocyte. The dashed line indicates the point at which NP_o is unchanged by alcohol.

Figure 6.

CaMKII increases wild-type bslo channel activity and switches channel responses to ethanol from activation to inhibition. (a,b) Current traces evoked at different potentials from excised I/O patches expressing wild-type bslo channels before (top), during (middle) and after (bottom) application of 100 mM ethanol to the cytosolic side of the patch. (a) Before CaMKII *in vitro* treatment, 100 mM ethanol reversibly increased wild-type bslo currents. (b) After CaMKII ($1,000 \text{ IU ml}^{-1}$ for 5 min) was applied to the same patch, ethanol inhibited wild-type bslo currents (shift in $V_{0.5} = -80 \text{ mV}$; 200-ms depolarizing steps from -40 mV to 160 mV , in 10-mV increments). Steady-state average current amplitude was determined 175–200 ms after the beginning of the depolarizing voltage step.

(c,d) Averaged $G/G_{\max}$ versus voltage data were fitted to a Boltzmann function. These fits show that both activated CaMKII and ethanol modified wild-type bslo currents by producing a parallel shift in the $G/G_{\max}$ versus V relationship. The data ($n = 5$) were obtained under conditions and protocols identical to those used to evoke currents shown in a and b.

Figure 7.

CaMKII phosphorylation of Thr107 progressively switches channel responses to ethanol. **(a)** Current traces from tetramers made of wild-type bslo and bslo T107V Y315V combinations recorded before and after ethanol administration, after CaMKII treatment. The top four traces show that as the proportion of T107V Y315V subunits decreased, so did the ethanol-induced potentiation (shown on the right of the traces as a percentage of control values). A cartoon depicts channel stoichiometry; the Y symbol indicates that Thr107 is phosphorylated. Tetramer composition was estimated from unitary currents, as Y315V introduces resistance to block by external TEA (Supplementary Note). The top four pairs of records were obtained in 2 mM external TEA. The fifth pair shows wild-type bslo currents in the absence of TEA. Recordings were obtained from I/O patches in symmetric 130 mM K⁺ with [Ca²⁺]_{ic} ≈ 0.3 μM; V = +60 mV; arrows indicate the baseline.

(b) The bar graph shows averaged ethanol responses. As the number of phosphorylated Thr107 residues increased, ethanol potentiation decreased, finally shifting to inhibition in phosphorylated bslo homotetramers. The white histogram bar indicates that wild-type bslo data were obtained in the absence of TEA. The dotted line shows linear regression fit to ethanol potentiation ($r = 0.999$). **(c)** T107E partially mimics the effect of CaMKII phosphorylation on bslo responses to ethanol. T107E homotetramers (second column in **c**) responded to ethanol as heterotetramers having two phosphorylated Thr107 (third column in **b**) ($P > 0.05$). $^{\#}P < 0.001$ in comparison to bslo T107V. Statistical differences between means were determined by ANOVA followed by Bonferroni's test. In **b** and **c**, data are shown as mean $\pm$ s.e.m; the number under each histogram represents the number of patches or oocytes.

Table 1

Ethanol responses for different slo channel populations and treatments

Channel type and treatment	(NPo in ethanol/NPo in control) $\times 100$ (mean $\pm$ s.e.m.)	<i>n</i>
Mslo	196.3 $\pm$ 16.7	10
bslo (nonactivatable population)	76.9 $\pm$ 6.9 ^{a,b}	17
bslo (activatable population)	221.6 $\pm$ 11.7	9
mslo V86T (nonactivatable population)	78.7 $\pm$ 6.9 ^{a,b,c}	11
mslo V86T (activatable population)	185.5 $\pm$ 8.3	8
bslo T107V	238.8 $\pm$ 18.7	19
bslo (nonactivatable population) after AP	250.9 $\pm$ 28. ^{6d}	5
bslo (activatable population) after AP	242.3 $\pm$ 22.9	5
bslo after KN93 preincubation	242.3 $\pm$ 12.9 ^d	9
bslo after KN92 preincubation	84.6 $\pm$ 11.6 ^{b,e}	9
bslo T107V after KN93 preincubation	243.6 $\pm$ 12.9	8
bslo T107V after AP	229 $\pm$ 18.9	7

^a $P < 0.01$ (in comparison to mslo).^b $P < 0.001$ (in comparison to bslo after AP).^c $P < 0.001$ (in comparison to activatable mslo V86T).^d $P < 0.001$ (in comparison to nonactivatable bslo V86T).^e $P < 0.001$ (in comparison to bslo preincubated with KN93).

Statistical analysis was conducted with multiple-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. *n*, number of patches. AP, alkaline phosphatase.