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Cristina Velazquez-Marrero
University of Puerto Rico

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Large Conductance Voltage- and Ca\(^{2+}\)-gated Potassium (BK) Channel \(\beta 4\) Subunit Influences Sensitivity and Tolerance to Alcohol by Altering Its Response to Kinases*

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Cristina Velázquez-Marrero, Garrett E. Seale, Steven N. Treistman, and Gilles E. Martin

From the 5Brudnick Neuropsychiatric Research Institute, University of Massachusetts Medical School, Worcester, Massachusetts 01604 and the 6Institute of Neurobiology, University of Puerto Rico Medical Sciences Campus, San Juan, Puerto Rico 00901

Background: Large conductance voltage- and Ca\(^{2+}\)-gated potassium channel (BK) \(\beta 4\) subunit profoundly influences BK acute ethanol tolerance with both physiological and behavioral consequences.

Results: PKA, CaMKII, and phosphatases modulate BK, and influence its response to ethanol. The presence of \(\beta 4\) strongly regulates these responses.

Conclusion: The control of BK \(\beta 4\) of kinase modulation is critical to ethanol response.

Significance: The influence of \(\beta 4\) on kinase-mediated alcohol action provides insight into the molecular basis for alcohol tolerance.

Tolerance is a well described component of alcohol abuse and addiction. The large conductance voltage- and Ca\(^{2+}\)-gated potassium channel (BK) has been very useful for studying molecular tolerance. The influence of association with the \(\beta 4\) subunit can be observed at the level of individual channels, action potentials in brain slices, and finally, drinking behavior in the mouse. Previously, we showed that 50 mM alcohol increases both \(\alpha\) and \(\alpha\beta 4\) BK channel open probability, but only \(\alpha\) BK develops acute tolerance to this effect. Currently, we explore the possibility that the influence of the \(\beta 4\) subunit on tolerance may result from a striking effect of \(\beta 4\) on kinase modulation of the BK channel. We examine the influence of the \(\beta 4\) subunit on PKA, CaMKII, and phosphatase modulation of channel activity, and on molecular tolerance to alcohol. We record from human BK channels heterologously expressed in HEK 293 cells composed of its core subunit, \(\alpha\) alone (Insertless), or co-expressed with the \(\beta 4\) BK auxiliary subunit, as well as, acutely dissociated nucleus accumbens neurons using the cell-attached patch clamp configuration. Our results indicate that BK channels are strongly modulated by activation of specific kinases (PKA and CaMKII) and phosphatases. The presence of the \(\beta 4\) subunit greatly influences this modulation, allowing a variety of outcomes for BK channel activity in response to acute alcohol.

It has long been apparent that individuals differ widely in their propensity to alcohol abuse and addiction. The large conductance voltage- and Ca\(^{2+}\)-gated potassium channel (BK) has been very useful for studying molecular tolerance. The influence of association with the \(\beta 4\) subunit can be observed at the level of individual channels, action potentials in brain slices, and finally, drinking behavior in the mouse. Previously, we showed that 50 mM alcohol increases both \(\alpha\) and \(\alpha\beta 4\) BK channel open probability, but only \(\alpha\) BK develops acute tolerance to this effect. Currently, we explore the possibility that the influence of the \(\beta 4\) subunit on tolerance may result from a striking effect of \(\beta 4\) on kinase modulation of the BK channel. We examine the influence of the \(\beta 4\) subunit on PKA, CaMKII, and phosphatase modulation of channel activity, and on molecular tolerance to alcohol. We record from human BK channels heterologously expressed in HEK 293 cells composed of its core subunit, \(\alpha\) alone (Insertless), or co-expressed with the \(\beta 4\) BK auxiliary subunit, as well as, acutely dissociated nucleus accumbens neurons using the cell-attached patch clamp configuration. Our results indicate that BK channels are strongly modulated by activation of specific kinases (PKA and CaMKII) and phosphatases. The presence of the \(\beta 4\) subunit greatly influences this modulation, allowing a variety of outcomes for BK channel activity in response to acute alcohol.

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1 To whom correspondence may be addressed. E-mail: steven.treistman@ upr.edu.

2 To whom correspondence may be addressed. E-mail: Gilles.Martin@ Umassmed.edu.

3 The abbreviations used are: BK, large conductance voltage- and Ca\(^{2+}\)-gated potassium channel; hSlo, human \(\beta 0\)-I gene that encodes the pore-forming subunit of a large conductance Ca\(^{2+}\)-activated K\(^+\) channel; CaMKII, Ca\(^{2+}\)/calmodulin-dependent protein kinase II; NAcc, nucleus accumbens; 8-Br-cAMP, 8-bromo-cAMP; IP, immunoprecipitation.
BK β4 Influences Phosphorylation and Alcohol Tolerance

robustly potentiated by relevant concentrations of alcohol in a number of brain regions (17–19), including the nucleus accumbens (20), a key brain region involved in addiction to drugs of abuse, including alcohol (21).

In this article, we examine whether the role of β4 in controlling acute molecular tolerance could reflect the influence of β4 on the actions of kinases and phosphatases. Our focus on kinases stems from a number of studies showing that a broad spectrum of kinases, including PKA (22–27), PKG (23, 28, 29), Src (30, 31), and CaMKII (32–34) regulate α BK channels. The effects of these enzymes are complex, and vary with preparation type, recording mode, and the particular splice variant of the BK channel α subunit (e.g. Insertless, STREX). There is strong evidence that kinases are key in mediating the effects of alcohol within the nervous system (35, 36). Furthermore, there is evidence that CaMKII mediates BK channel sensitivity to alcohol in expression systems (33). Interestingly, evidence suggests that protein kinase C (PKC), cyclic AMP-dependent protein kinase A (PKA), calcium/calmodulin-dependent protein kinase II (CaMKII), protein kinase G (PKG), and G protein receptor kinase (GRK) can act as molecular switches to modulate opioid tolerance (37).

We first examined the influence of PKA, calcium/calmodulin kinase II (CaMKII), and phosphatases (PP) on the effects of alcohol on the BK channel containing α (Insertless isoform, or IL) and β4 subunits in HEK 293 cells. We then examined the regulation of EtOH-mediated potentiation of BK channels by these kinases in nucleus accumbens neurons. We found that the presence of the β4 subunit dramatically alters the effects of PKA, CaMKII, and PP, and that these effects of β4 were consistent with a role in the modulation for β4 of acute tolerance.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection Techniques—Our methods are essentially the same as those outlined in Feinberg-Zadek and Treistman (38). Briefly, hSlo channels were derived from two stable cell lines. The HEK α-1.2 cell line (a gift from Peter Ahring (39)) stably expresses the human BK channel α-subunit splice variant, hbr1 derived from brain (39, 40). The BK channels expressed in this cell line will be referred to as hSlo. The hSlo αβ4 channels were derived from cell lines stably expressing hSlo and transiently expressing hβ4 (human β4 subunit, GenBank accession number AF215891). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 50 units/ml of penicillin, 50 mg/ml of streptomycin, and 2.5 mM HEPES (Invitrogen) at 37 °C in humidified 5% CO2 incubator. Cells were transfected with kinases stems from a number of studies showing that a broad on the actions of kinases and phosphatases. Our focus on

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Freshly Dissociated Nucleus Accumbens Neurons—This method is described in detail in Martin and Siggins (41), and we will briefly summarize it here. After decapitation of Sprague-Dawley rats (80–150 g), rat brains were rapidly transferred into a cold (4 °C) oxygenated, low-calcium HEPES-buffered salt solution to be sliced (400 μm thick) using a vibraslicer (Vibratome 3000). Slices were incubated for up to 6 h at room temperature (20–22 °C) in a gassed (95% O2 and 5% CO2) NaHCO3-buffered saline solution. After 1 h of incubation, we dissected out the region of the nucleus accumbens and incubated the tissue for 25 min in an oxygenated (100% O2 with constant stirring) HEPES-buffered solution in the inner chamber of a Cell-Stir flask (Wheaton, Millville, NJ) containing protease XIV (1 mg/ml). After mechanical trituration of the tissue using fire-polished Pasteur pipettes, we plated the supernatant into a 35-mm Petri dish placed on the stage of an inverted microscope (Axovert 200, Zeiss Germany). The cells were allowed to attach to the dish for 10 min before replacing the Na+-isethionate solution with normal external solution flowing at a rate of 1.5 ml/min.

Bathing Solutions—For the subsequent duration of the experiment, a modified PO4-free Hank’s solution (in mM: NaCl 120; KCl 10; MgCl2 2; CaCl2 2.2; glucose 5; HEPES 10) was perfused through the recording dish. During excised inside-out patch recordings the bathing solution also contained 1 mM ATP. Kinase and phosphatase inhibitors and activators were prepared as ×1000 stock solution, diluted in 20 ml of background solution in 50-ml syringes and expelled from hemocrit tubes. Pipette tips were positioned in the “mouth” of the hemocrit tubes to prevent contamination from solution potentially leaking from nearby tubes. When we tested the effects of PKA, PKC, CaMkII, and phosphatase inhibitors (Sigma) on the effects of EtOH, we also added these drugs in the background solution running through the recording chamber so that cells were exposed at least 10 min before exposing cells to EtOH.

Electrophysiological Recordings—We used both the standard single-cell cell-attached patch clamp recording method (42) and the excised inside-out patch clamp configuration. Briefly, we pulled and fire-polished patch electrodes from 1.5-mm OD borosilicate capillary glass (Warner Instrument, CT) on a P-97 Brown-Flaming puller (Sutter Instruments) to a final resistance of 4–6 MΩ for. The recording pipette solution was (in mM): 130 K2MeSO4, 2 MgCl2, 2 CaCl2, 15 HEPES. The capillaries were first filled through the tip and then backfilled with the recording solution. We recorded BK channel currents at a sampling rate of 10 kHz and low-pass filter of 2 kHz with an EPC10 double amplifier (HEKA Electronics, Germany). Voltage and currents were digitized and stored using PatchMaster 2.1 acquisition (HEKA Electronics, Germany) running on a PowerPC G5 (Apple computer). All amplitude histograms were obtained with TAC 4.1.5, single channel analysis software (Bruxton, WA) running on an iMac G5 (Apple computer, CA). We applied no leak substitution when we evoked currents using a step protocol. Data were sampled for a period of 15–20 min. As a control, we recorded BK channel activity for 10 s, every minute, three times to ensure a stable baseline activity. We averaged the open probability of the three controls, and all control and drug NPo values were expressed as percent relative to this average value. All results are expressed as mean ± S.E. values. Drugs were applied and BK channel activity was recorded in successive blocks of 10 s, every minute, for up to 10 min. Maximal EtOH effects during this period were used in the analysis, independent of the time of exposure. Data were expressed as mean ± S.E. (with the number of cells or patches in parentheses). When testing PKA, CaMkII, and PP inhibitor effects on EtOH-mediated potentiation of BK channel activity, these drugs were added in the main superfusing solution minutes before recordings to ensure that all cells were thoroughly exposed to the drugs. We also added these inhibitors in both the control and EtOH containing syringes connected to capillaries places in proximity of HEK cells while recording to ensure that cells were constantly exposed to inhibitors throughout the experiments.

Charybdotoxin Treatment and αβ4 BK Channels—Because hSlo αβ4 channels are insensitive to low concentrations of charybdotoxin, an α channel pore blocker that inhibits activity of hSlo α channels very rapidly at 100 nm (13), we added this toxin in the recording electrode solution to ensure that only channels containing the β4 subunit were recorded.

Calculation of the Steady-state Channel Activity, NPo—Calculation of steady-state channel activity was determined from the product of the total number of functional channels present in the membrane patch (N) and the probability that a particular channel was open under steady-state conditions (Po). For the construction of the histograms and the idealized records, data were obtained during a continuous gap-free recording period for a total time of no less than 20 s. The Po was obtained from patches held between +20 and +40 mV. For each patch, BK channel activity was recorded for 60 s every minute for the whole duration of the recordings. The first control period was determined from the 30-s drug-free period immediately prior to exposure. All NPo ratios generated for the first exposure used this control for normalization of the data. BK channel activity was measured as NPo ratio percent ((NPo/NPo control) × 100). The calculations of NPo were performed using TAC analysis software (Bruxton Inc., OR).

Statistical Comparisons—In all cases, data are reported as mean ± S.E.; n being the number of cells or neurons. Statistical analysis of difference were made with paired t test, with p < 0.05 considered significant.

RESULTS

BK channel activity was stable over the 20–25-min recording period, assuring that changes in open probability following drug application could be attributed to the drug, and not to random changes in channel activity. BK hSlo channel activity in transfected HEK cells was elicited in cell-attached patch clamp mode with depolarizing voltage steps. Because α and αβ4 have very different I-V relationships (13, 14), it was not possible to compare effects of the various kinases and EtOH on channel activity at similar holding potentials. Therefore, we held membranes at a potential where the resulting channel activity would allow both inhibition and potentiation to be observed. Fig. 1A shows representative traces and Fig. 1B the averaged open probability of BK channels from three different membrane
**BK β4 Influences Phosphorylation and Alcohol Tolerance**

**Figure 1. Acute effects of EtOH on α and αβ4 BK channels in HEK 293 cells.** A, traces of the same α BK channel at different time points (between 1 and 15 min). Currents were evoked by depolarizing the membrane potential to +110 mV from a holding of −30 mV, as represented by the square pulse, below traces. The numbers above the traces indicate open probability. B, averaged BK channel activity from 3 patches measured every minute for nearly 20 min. The dashed line shows the baseline control level. All % of baseline described values are expressed as percent change compared with control (first three measurements). C, representative traces of an α BK channel activity before (control) and during EtOH exposure (2 and 7 min). Open probability is indicated by the numbers below the traces. D and E, averaged BK channel activity before and during EtOH exposure for α (D) and αβ4 (E) BK channel. The dashed line shows the baseline control level. All % of baseline describes values are expressed as percent, change compared with control value (100%). F, representative traces of an αβ4 BK channel activity, recorded before (control) and during EtOH exposure (2 and 7 min) as previously published (11) presented to facilitate comparisons. The open probability is indicated by the numbers below the traces. Asterisks represent statistically significant differences from baseline: * indicate $p \leq 0.05$.

Response to Acute Alcohol Challenge—We replicated previous findings that β4 effectively blocks the development of acute molecular tolerance in BK (11). The response of the α BK channel to 50 mM EtOH was characterized by an initial increase of open probability from 0.057 to 0.142 at 2 min (Fig. 1C). This response was followed by a rapid return of open probability to pre-exposure levels, illustrated with representative data from a single experiment (NPo = 0.051; Fig. 1C, EtOH 7 min), and as an average from multiple recordings in Fig. 1D (darker gray box). As with α BK, αβ4 BK channel open probability increased (from 0.10 to 0.41 Fig. 1F, EtOH 3 min) in the presence of 50 mM EtOH. However, in stark contrast to α alone, the αβ4 BK channel displayed no tolerance, and NPo remained elevated in the continued presence of EtOH (Fig. 1F, EtOH 8 min). When averaged over 6 patches, EtOH potentiated αβ4 BK channel activity by 2.56 ± 0.43-fold (Fig. 1E), and this potentiation was undiminished 8 min after alcohol exposure (Fig. 1E, darker gray box, $p < 0.05$).

β4 Regulates Kinase Modulation of BK—Having confirmed the effect of β4 on acute molecular tolerance, we next explored whether kinase modulation of activity differed in α and αβ4 BK channels.

Protein Kinase A (PKA) Pathway—We examined the PKA pathway using 250 μM 8-Br-cAMP, a membrane permeable selective PKA inhibitor. Fig. 2A shows representative traces
illustrating the increase of a BK activity 6 min after superfusion of 8-Br-cAMP (top trace). Averaged over 5 patches, activity was clearly increased 2 min after 8-Br-cAMP superfusion, reaching steady-state after 5 min (Fig. 2C). When repeated with HEK cells co-expressing α and β4 BK subunits, 250 μM 8-Br-cAMP, unlike for α BK alone, failed to potentiate αβ4 BK activity in a statistically significant manner (Fig. 2, B and D). These results strongly suggest that the ability of PKA to directly or indirectly phosphorylate BK channels is modulated by the presence of the β4 subunit. We further tested the specificity of the PKA activation of α BK by co-incubating with 100 nM PKA-(14–22), a membrane permeable PKA inhibitor (Fig. 3). The averaged responses over 10 min after application of 8-Br-cAMP resulted in an increase of 2.57 ± 0.46 times over control (n = 4) (Fig. 3A). Adding the PKA inhibitor blocked the increase, resulting in an NPo, statistically unchanged from non-treated (0.86 ± 0.1219, n = 4) (Fig. 3A). Therefore, activation resulting from 8-Br-cAMP is likely dependent upon cAMP-dependent protein kinase activity. Immunoprecipitation of α BK transiently expressed in HEK 293T cells also corroborates direct phosphorylation of the α BK channel complex by PKA. Quantification of normalized phospho-PKA substrate immunoblot intensity of anti-HA IPs from control, 8-Br-cAMP, and 8-Br-cAMP + PKA1 Asterisks represent statistically significant differences: ** indicates p < 0.01 and * indicates p < 0.05.

Interestingly, when α BK channels were treated with 100 nM PKA-(14–22) alone, activity was nearly unchanged, as shown by representative individual traces in Fig. 2E (0.019 before and 0.003 min after exposure to PKA-(14–22)), and by the averaged NPo, of BK channels in patches from 6 different cells (Fig. 2G). These results suggest that α BK channels are not constitutively activated by PKA under basal conditions in HEK cells. Although 8-Br-cAMP, the selective PKA activator, had no effect on αβ4 BK channel activity (Fig. 2, B and D), we never-
theless examined the effect of PKA-(14–22) (Fig. 2, F and H), which did not produce a statistically significant change in BK channel open probability.

To explore the mechanism of PKA activation, we assayed the functional regulation of BK channels via PKA utilizing expression of point-mutated cDNA constructs on a specific PKA regulation site within the BK subunit. Tian and colleagues (43), demonstrated that the BK channel requires a conserved C-terminal PKA consensus motif known as Ser-869 to be activated by PKA. By creating a single point mutation of the serine 869 to alanine (S869A) they abolished the cAMP-mediated activation of BK channels, also known as the ZERO isoform. Thanks to their generosity, we were able to test the previously described ZERO-S869 control (ZERO) and ZERO-S869A variant, with and without β subunit expression. Once these channels were expressed in HEK 293 cells, we recorded channel sensitivity to PKA in the inside-out patch clamp configuration. This experimental approach affords us two main advantages to test the mechanism of PKA modulation. First, we are able to directly activate PKA, which remains closely associated with the channels, in excised inside-out patches (Fig. 4, A and B) (44). Thus, we can then determine whether activation occurs within the channel complex, further specifically associated to the 869 site within the BK channel. Second, recording from excised inside-out patches we are able to study PKA activation isolated from the effects of other kinases on the channel complex such as CaMKII (32). This is particularly important given we have found CaMKII has a tonic effect on BK channels when recording from cell-attached patches (Fig. 5).

Our results indicate that application of 0.1 mM cAMP to the intracellular face of excised inside-out patches containing either α and αβ4 BK channels resulted in significant (p < 0.05) activation of mean channel activity in all patches (n = 5). The mean percentage activation in response to cAMP was 182.34 ± 21.93% for ZERO (α BK) and 295.29 ± 41.93% for ZERO β4 (αβ4 BK) (Fig. 4, A and B). As previously observed, channel

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**FIGURE 4.** Effect of PKA activation on α and αβ4 BK channel N\_P0 in the wild-type channel and in channel expressing S869A mutation in HEK 293 recorded using excised patches. A, bar graph quantifying N\_P0 of individual inside-out patch clamp recordings with 0.1 mM cAMP in the bath solution containing 1 mM ATP. The graph is plotted showing PKA activation as percent of control (untreated). The dashed line shows the baseline control level. B, representative traces of α (hp = +40 mV) and αβ4 (hp = +20 mV) BK channel activity before (control) and during 0.1 mM cAMP exposure (6 min). The numbers below the traces indicate open probability. Number of patches tested, one patch per cell, in each condition was n = 4. Asterisks represent statistically significant differences: *, p ≤ 0.05, and **, p ≤ 0.01.

**FIGURE 5.** Effects of inhibiting CaMKII on α and αβ4 BK channel open probability. A and B, sample traces of BK channel activity before and during drug exposure. The right-hand side of each trace shows the N\_P0 measured during 20 s recording. C and D, average BK channel activity before (control) and during drug exposure (filled bars). The dashed line shows the baseline control level. Number of patches tested in each condition is indicated by the n value shown in the lower left part of the graphs. Asterisks represent statistically significant differences from baseline: * indicates p ≤ 0.05, and ** indicates p ≤ 0.01.
activation was dependent upon a conserved C-terminal PKA consensus motif (Ser-869) where, mutation of serine 869 to alanine (S869A) completely abolished cAMP-mediated activation of αBK channels (the ZERO variant). Corroborating these earlier results, the ZERO S869A construct resulted in a mean ± change activity in response to cAMP of −0.02 ± 7.48% (n = 5) (Fig. 4A). These data, in addition to highlighting the actions of PKA, further confirm previous results indicating that Ser-869 is essential for PKA-mediated activation of the αBK channel (44).

Interestingly, αβ4 BK channels expressing the same S869A mutation exhibited a marked cAMP-mediated increase in activity (169.00 ± 16.72%), in clear contrast to the αBK S869A channels (Fig. 5A). These data suggest that the β4 subunits either have sites directly phosphorylated by PKA, which result in an additive/synergistic effect with endogenous αBK activation, or that the β4 association fundamentally changes how PKA interacts with BK channels, possibly favoring PKA phosphorylation sites other than Ser-869 on the αBK subunit (see "Discussion").

**CaMKII Pathway**—Following the same protocol as above (3 min control recording followed by a 10 min drug application), we examined the CaMKII pathway. CaMKII (100 nM), a membrane-permeable selective CaMKII inhibitor markedly increased αBK channel activity. Fig. 5A shows representative records of αBK activity, which increased nearly 2.5-fold when compared with pre-treatment (control, 0.21; 3 min post-CaMKII, 0.55). The increase, after averaging αBK channel activity from five different membrane patches, is shown in Fig. 5C. These data suggest that αBK channels are tonically inhibited by CaMKII. In stark contrast, the αγ4 BK channel was markedly attenuated by CaMKII, reduced from an N\(_{P_0}\) of 0.34 to 0.01 (Fig. 5B). When averaged over 6 patches, it is apparent that this effect developed gradually, reached a peak (about 40% of control) in 5 min, and remained steady for the following 5 min (Fig. 5D). As with the PKA pathway, these results clearly illustrate the extraordinary influence of the β4 subunit on the modulation of the channel by kinases.

**Phosphatases**—Because the actions of protein kinases are typically balanced by that of protein phosphatases, we examined the effects of 1 μM okadaic acid, a potent phosphatase (PP1 and PP2A) inhibitor. As illustrated in Fig. 6, A and C, αBK channel activity increased by more than 3-fold (to N\(_{P_0}\) = 0.095) 6 min after OA exposure, compared with pre-treatment (N\(_{P_0}\) = 0.03). This potentiation is visible 1 min after okadaic acid exposure and increases steadily to reach a plateau after 7–8 min (Fig. 6C). In contrast, we found that 1 μM okadaic acid markedly attenuated αγ4 BK channel activity. Thus, Fig. 6, B and D, show that the open probability of this channel (0.28 pre-treatment; Fig. 6B, top trace) decreased to 0.09, 5 min after okadaic acid superfusion (Fig. 6B, bottom trace). On average (6 patches), okadaic acid attenuated αγ4 BK activity by about 64% (Fig. 6D).

Thus, co-expression of the β4 subunit reverses the effects of phosphatase inhibition observed with the αBK subunit alone.

We next examined the influence of β4 on activation of phosphatase activity. Activation of PP2A by 30 μM N-hexanoyl d-erythro-sphingosine increased αBK channel activity in 5 cells by about 150% (Fig. 6F), whereas application to αBK channels had no statistically significant effect (Fig. 6E).

**FIGURE 6. Effects of okadaic acid (OA) and protein phosphatase A\(_2\) activator N-hexanoyl-o-erythro-sphingosine (NHDS) on αBK and αγ4 BK channel activity.** A and B, sample traces of BK channel activity before and during drug exposure. On the right-hand side of each trace is the N\(_{P_0}\) measured during 20 s recording. C and D, average BK channel activity before (control) and during okadaic acid exposure (filled bars). The dashed line shows the baseline control level. E and F, same as C and D, with NHDS, instead of okadaic acid. Number of patches tested in each condition is indicated by the n value shown in the lower left part of the graphs. Asterisks represent statistically significant differences from baseline: * indicates \(p \leq 0.05\).

Clearly, β4 has a profound influence on the actions of kinases and phosphatases on BK. We next examined how this influence of β4 extended to kinase-mediated effects of ethanol on the channel.

**Role of β4 in Kinase-mediated EtOH Regulation of BK**—Having demonstrated the powerful influence of β4 on the modulation of BK activity by PKA, CaMKII, and PP, we examined their role in mediating the effects of EtOH on BK. The protocol was to incubate HEK cells for several minutes with the antagonists before introducing 50 mM EtOH. We chose this concentration because it leads to the largest BK channel potentiation in both HEK cells (38) and neurons (20). As previously shown in Fig. 1, C and D, EtOH-mediated potentiation of αBK channels is transient, typically peaking after 2–3 min (potentiation, light gray area, Fig. 1, C and D), and returning to pre-drug exposure levels within 5–7 min (rapid tolerance; darker gray area, Fig. 1D). αβ4 BK channels had been previously shown to exhibit increased (about 5-fold) channel activity in response to EtOH (38), in inside-out patch clamp recording mode. Here, we confirmed that 50 mM EtOH also potentiated αβ4 BK channel activity in the cell-attached mode (Fig. 1, E and F), although the magnitude...
of this effect was smaller, a difference that may reflect different free calcium concentrations in the intact cell compared with recordings from inside-out excised patches (38).

Effect of the PKA Pathway on BK Response to EtOH—We examined the effect of the PKA inhibitor, PKA-(14–22) (100 nM) on ethanol-mediated potentiation and tolerance of α BK channels. Ethanol-mediated enhancement of α BK channel activity was completely blocked by PKA-(14–22) pretreatment (Fig. 7A, light gray area), suggesting that potentiation of α BK channels by ethanol may be mediated by PKA. We then examined the role of PKA in mediating the potentiation of αβ4 BK channel activity and its lack of tolerance. We found that channel potentiation was sustained over the 10-min recording period following exposure (data not shown), unlike what we observed in the absence of okadaic acid (Fig. 1D). This demonstrates that phosphatases can influence ethanol-mediated tolerance of α BK channels. The effects of ethanol after pre-exposing αβ4 BK channel to okadaic acid showed no net change in BK channel activity (data not shown).

Ethanol Effects on αβ4 BK Channels in NAcc Medium Spiny Neurons—Data obtained from HEK cells, as described above, suggest a role for CaMKII-mediated channel phosphorylation in the block of acute tolerance by β4, and we began with this finding to extend our studies from HEK cells to neurons. We examined whether a CaMKII inhibitor could induce acute tolerance in BK channels from NAcc medium spiny neurons, which normally do not show this attribute (33). Rat NAcc medium spiny neurons express two BK channel subtypes, αβ1 and αβ4 (20). Somatic BK channels were recorded in the cell-attached patch clamp mode in the presence of low (100 nM) concentrations of charybdotoxin in the recording pipette. This toxin ensured that all α or αβ1, but not αβ4, BK channels were blocked (13, 45, 46). As illustrated in Fig. 8, A and B, NAcc αβ4 BK channels, like their counterpart in HEK 293 cells, were potentiated by 50 mM EtOH, and this effect persisted over the duration of the recording (i.e., about 10 min). The potentiation and the lack of tolerance are clearly visible in the graph of averaged NAcc BK channel activity before and during exposure (Fig. 8B). The maximum increase of αβ4 BK channel NP (2.2-fold) in the NAcc was close to that of HEK 293 cells (2.5-fold). We then examined the role played by CaMKII in mediating acute effects of EtOH in these neurons. BK channel activity increased 1–3 min after the beginning of alcohol exposure (Fig. 8C). However, this increase was not sustained and soon returned to control levels (Fig. 8C, lower trace). When averaged (n = 4), BK channel activity peaked (2.4-fold increase) after 2 min before returning to pre-exposure levels within 6 min (Fig. 8D). Thus, inhibition of CaMKII does, indeed, induce acute tolerance in BK channels from NAcc medium spiny neurons, extending these findings from those observed in transfected HEK cells.
absence of the β4 subunit, there is no activation of the BK channel as previously reported (43). Thus, PKA activation is via phosphorylation directly within the channel. We further show that when the normally PKA-insensitive variant α(S869A) BK channel is associated with the β4 subunit, there is significant cAMP-mediated activation of the α(S869A)β4 BK complex channel. Interestingly, the association of the α BK channel with its β4 subunit opens new possibilities for PKA regulation that are independent of the Ser-869 α subunit site. In fact, the non-mutated PKA-sensitive α(Ser-869) BK, when associated to β4, responds by potentiating its PKA activation. These results highlight the possibility of independent sites for phosphorylation made accessible through mechanical reconfiguration of the α subunit as a result of its association with β4 and/or sites on the β4 that may be further modulated by PKA directly. The β4 subunit itself contains three serine-binding motifs in its protein sequence that may serve as putative PKA and/or CaMKII phosphorylation sites (Table 1). Thus, these putative binding motifs could mediate the increase in activation of the BK channel kinases, phosphatases, and BK channels are tightly controlled by the β4 BK channel auxiliary subunit. Second, these kinases mediate, at least partially, the response of the BK channel to ethanol, and this mediation is also β4-dependent. Our data showing potentiation of BK channel α subunit activity by PKA confirms the findings in a number of studies. Earlier work by Kume et al. (47) and others (48, 49) reported a PKA-mediated increase in BK channel open probability. However, lack of information regarding subunit composition makes it difficult to draw a strict parallel with our own study. Dworetzky et al. (50) found that PKA increased the open probability of BK channel α subunit expressed in HEK 293 cells. Interestingly, Tian et al. (43) found that the ability of PKA to boost α BK activity was associated with a specific site (Ser-869) on the BK channel α subunit lacking splice inserts, known as ZERO or Insertless, which is the α subunit isoform examined in the present work. We are currently exploring the mechanisms of PKA activation and the role of this conserved C-terminal PKA consensus motif (Ser-869) when α BK is associated with the β4 subunit. These experiments using excised inside-out patches of HEK 293 cells, transiently expressing either the control (Ser-869) or the PKA-insensitive variant (S869A), show two main points. First, that association with the β4 subunit changes the dependence of α BK on the Ser-869 site for PKA activation. Second, PKA modulation of both α and αβ4 BK does occur within the channel complex. These experiments corroborate that when the C-terminal site is mutated (S869A), in the absence of the β4 subunit, there is no activation of the BK channel as previously reported (43). 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Thus, these putative binding motifs could mediate the increase in activation of the BK channel

### DISCUSSION

This study provides two key new findings. First, interactions between kinases (i.e. PKA and CaMKII), phosphatases, and BK channels are tightly controlled by the β4 BK channel auxiliary subunit. Second, these kinases mediate, at least partially, the response of the BK channel to ethanol, and this mediation is also β4-dependent. Our data showing potentiation of BK channel α subunit activity by PKA confirms the findings in a number of studies. Earlier work by Kume et al. (47) and others (48, 49) reported a PKA-mediated increase in BK channel open probability. However, lack of information regarding subunit composition makes it difficult to draw a strict parallel with our own study. Dworetzky et al. (50) found that PKA increased the open probability of BK channel α subunit expressed in HEK 293 cells. Interestingly, Tian et al. (43) found that the ability of PKA to boost α BK activity was associated with a specific site (Ser-869) on the BK channel α subunit lacking splice inserts, known as ZERO or Insertless, which is the α subunit isoform examined in the present work. We are currently exploring the mechanisms of PKA activation and the role of this conserved C-terminal PKA consensus motif (Ser-869) when α BK is associated with the β4 subunit. These experiments using excised inside-out patches of HEK 293 cells, transiently expressing either the control (Ser-869) or the PKA-insensitive variant (S869A), show two main points. First, that association with the β4 subunit changes the dependence of α BK on the Ser-869 site for PKA activation. Second, PKA modulation of both α and αβ4 BK does occur within the channel complex. These experiments corroborate that when the C-terminal site is mutated (S869A), in the absence of the β4 subunit, there is no activation of the BK channel as previously reported (43). Thus, PKA activation is via phosphorylation directly within the channel. We further show that when the normally PKA-insensitive variant α(S869A) BK channel is associated with the β4 subunit, there is significant cAMP-mediated activation of the α(S869A)β4 BK complex channel. Interestingly, the association of the α BK channel with its β4 subunit opens new possibilities for PKA regulation that are independent of the Ser-869 α subunit site. In fact, the non-mutated PKA-sensitive α(Ser-869) BK, when associated to β4, responds by potentiating its PKA activation. These results highlight the possibility of independent sites for phosphorylation made accessible through mechanical reconfiguration of the α subunit as a result of its association with β4 and/or sites on the β4 that may be further modulated by PKA directly. The β4 subunit itself contains three serine-binding motifs in its protein sequence that may serve as putative PKA and/or CaMKII phosphorylation sites (Table 1). Thus, these putative binding motifs could mediate the increase in activation of the BK channel

### Table 1

Predicted PKA and CaMKII phosphorylation sites on the KCNMB4, human calcium-activated potassium channel subunit β4 protein sequence.

| Position in Protein | Sequence in Protein | Corresponding motif described in the literature | Features of motif described in the literature |
|---------------------|---------------------|----------------------------------------------|------------------------------------------|
| 1                   | SR                  | PKA kinase substrate motif                    |                                          |
| 2                   | RGT                 | PKA kinase substrate motif                    |                                          |
| 3                   | RGT5                | Calmodulin-dependent protein kinase II substrate motif |
| 4                   | RGT5                | PKA kinase substrate motif                    |                                          |
| 5                   | RGT5                | Calmodulin-dependent protein kinase II substrate motif |
| 6                   | KE5                 | PKA kinase substrate motif                    |                                          |
| 7                   | KN5                 | PKA kinase substrate motif                    |                                          |
| 8                   | RX5                 | PKA kinase substrate motif                    |                                          |
| 9                   | RX5                 | Calmodulin-dependent protein kinase II substrate motif |
| 10                  | RX5                 | PKA kinase substrate motif                    |                                          |
| 11                  | RX5                 | Calmodulin-dependent protein kinase II substrate motif |
| 12                  | RX5                 | PKA kinase substrate motif                    |                                          |
| 13                  | RX5                 | PKA kinase substrate motif                    |                                          |
| 1                  | MQLRVAYEY         | PKA kinase substrate motif                    |                                          |
| 2                  | MLQIVYEGWV        | PKA kinase substrate motif                    |                                          |
| 3                  | MWKVQWF            | PKA kinase substrate motif                    |                                          |
| 10                 | EANTVLSQV       | PKA kinase substrate motif                    |                                          |
| 151                | QAMLSPDDV        | PKA kinase substrate motif                    |                                          |
| 201                | AEAMKKCFGKS      | PKA kinase substrate motif                    |                                          |

Note: The table lists predicted PKA and CaMKII phosphorylation sites on the KCNMB4, human calcium-activated potassium channel subunit β4 protein sequence. The sites are predicted using the PhosphoMotif Finder (60), and the features of the motifs are described in the literature. The table includes positions, sequences, corresponding motifs, and features of the motifs described in the literature.
that CaMKII tonic endogenous activity may prevent PKA modulation (Fig. 5, C and D) in the presence of β4 either by changing the configuration of sites phosphorylated within the β4 subunit and/or newly accessible sites on the α subunit itself. Whether CaMKII is blocking PKA activation or saturating the channels activation response remains to be explored. However, the interplay between the endogenous profile of kinase/phosphatase activity and their inherent affinities for specific phosphorylation sites within both α and αβ4 BK channel complexes are likely complexly interlinked and ultimately will determine the channels physiological response.

It is notable that other studies (51, 52), performed on GH4 cells, reported that PKA inhibited BK channel activity. These divergent results may originate from different BK channel α subunit isoforms (10) being studied. Supporting this idea, the activity of α subunits expressing a 59-amino acid exon called STREX (stress axis hormone-regulated exon), was inhibited by PKA (53). The possibility that PKA-mediated potentiation of α subunit activity by EtOH may be physiologically relevant is supported by the fact that EtOH elevates PKA levels in neurons (35, 36). It is possible that PKA effects on the BK channel are mediated via direct interaction with the channel, as demonstrated by the BK functional LZ1 domain co-immunoprecipitation with a PKA-signaling complex in rat brain (26) and our current results corroborating the importance of the Ser-869 site within the α BK channel (Fig. 4A) (43).

Previous studies (32, 34) document the role of CaMKII in regulation of BK channel activity, reporting increased channel activity. In glomerular mesangial cells, CaMKII activation by contractile agonist ANGII potentiates BK channel activity (54). Liu et al. (33) reported that incremental CaMKII-mediated phosphorylation of Thr-107 in the α BK tetramer progressively increased channel activity and gradually switched channel alcohol responses from robust activation to inhibition. They hypothesized that CaMKII phosphorylation of Slo Thr-107 could mediate tolerance to alcohol. Our results show a robust potentiation of the α BK channel and an inhibition of the αβ4 BK channel in the presence of the CaMKII inhibitor alone. Putative CaMKII sites in the intracellular domain of the β4 subunit (Table 1) may be responsible for activation of the αβ4 BK channel complex that would not be otherwise present. These sites when phosphorylated may increase αβ4 BK channel activity and thus be inhibited when CaMKII is inhibited. Future studies will focus on determining the role of these phosphorylation sites and BK channel modulation.

Most interestingly, the present study clearly demonstrates the ability of the β4 subunit to dramatically alter, not only PKA and CaMKII modulation of BK channels, but also their role in mediating the effects of EtOH. Notably, PKA potentiation of BK is completely blocked by β4 subunit expression and in the presence of the β4 subunit, PKA modulation of the BK channel is no longer necessary for potentiation by alcohol. However, for CaMKII, the β4 subunit plays a subtler role as it does not block the effects of CaMKII but rather alters them. Moreover, our results suggest a role for CaMKII-mediated phosphorylation in the block of acute tolerance by β4.

Possibly relevant to our results are published data examining the role of phosphorylation in mediating the effects of the β4 subunit on BK channel activation and deactivation kinetics (55). Jin and colleagues (55) concluded that phosphorylation of different residues in αβ4 differentially influences its effects on hSlo channel activation kinetics, deactivation kinetics, and voltage dependence. As mentioned, it is certainly possible that the effects we observe could be due to phosphorylation of the β4 subunit, subsequently modulating α BK channel activity. Alternatively, association with the β4 subunit may influence phosphorylation of the α BK channel itself. In colonic myocytes, BK channel modulation via PKC requires association with the β1 subunit, likely attributable to changes in BK channel conformation when associated with the β1 subunit (56). These authors propose that association with β1 selectively unmasks PKC phosphorylation sites on the α BK subunit, thus influencing PKC sensitivity. Mechanistically, the reduced influence of PKA we observe in BK channels in the presence of the β4 subunit suggests that the auxiliary subunit may either prevent PKA from accessing specific phosphorylation sites, or more radically, may physically uncouple PKA from BK channels. Additionally, it is possible that PKA is able to access BK in the presence of β4, but the consequences of this interaction are altered by β4. At this point, the mechanism(s) underlying β4 modulation of BK channel interaction with PKA and CaMKII are unclear.

A striking conclusion emerging from recent work on alcohol sensitivity and molecular tolerance is the fact that a single gene product (the α BK subunit) can exhibit radically different alcohol responses, based upon post-transcriptional events (10) (Fig. 9). Thus, influences such as: 1) microRNA mediated stability of splice variants of the channel protein (57), 2) association with auxiliary subunits such as β4 (11, 38) (and results presented here), and 3) lipid environment (9, 38, 58, 59) produce BK that
differ in their immediate response to alcohol and in their propensity to develop acute tolerance, to a degree that belies their origin from a single gene.

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