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Large Conductance Voltage- and Ca^{2+}-gated Potassium (BK) Channel β4 Subunit Influences Sensitivity and Tolerance to Alcohol by Altering Its Response to Kinases*

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Background: Large conductance voltage- and Ca^{2+}-gated potassium channel (BK) β4 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 β4 strongly regulates these responses.
Conclusion: The control of BK β4 of kinase modulation is critical to ethanol response.
Significance: The influence of β4 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 β4 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 α and $\alpha\beta$4 BK channel open probability, but only α BK develops acute tolerance to this effect. Currently, we explore the possibility that the influence of the β4 subunit on tolerance may result from a striking effect of β4 on kinase modulation of the BK channel. We examine the influence of the β4 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, α alone (Insertless), or co-expressed with the β4 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 β4 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 to becoming addicted to alcohol. Escalation of drug use in animal models can be manipulated by both selective breeding and selected drug exposure protocols (1–4). As with all behavior, we can assume that these behavioral phenomena reflect differences in molecular physiology and pharmacology within the nervous system. Alcohol tolerance is a decreased response to the functional effects of alcohol, subsequent to previous exposure to the drug. Reduced sensitivity to alcohol may lead to higher consumption and is a key factor in initiation and maintenance of alcohol dependence (5, 6). Moreover, the magnitude of acute behavioral tolerance observed in individuals can indicate a predisposition to alcohol abuse and addiction in human beings (7, 8). The large conductance voltage- and Ca^{2+}-gated potassium channel (BK)[3] channel has proven to be a very useful model for understanding the basis of acute tolerance at the molecular level (9–11). Recent work using genetically manipulated mice in which the BK channel β4 subunit has been "knocked out" (KO) suggests remarkable parallels in the effects of the β4 subunit on acute alcohol tolerance at the levels of single channel recording, spike patterning, and behavior (11). At each level, acute tolerance was apparent within a few minutes in β4 KO mice, but not wild-type (WT) mice. Moreover, the β4 KO mice voluntarily drank significantly more alcohol than WT mice (11). Thus, the predictive value of acute tolerance observed in humans has been replicated in the rodent model, and a potential genetic mediator identified.

BK channels are large conductance potassium channels expressed throughout the brain, where they exist as a complex of subunits, including the pore forming α subunit, which is the product of a single gene, and four regulatory β subunits, products of four distinct genes. Although the α BK subunit is ubiquitously expressed in the brain (12), only β1 and β4, have been reported in neurons of the central nervous system (13, 14). Evidence suggests that BK channels are critical in alcohol-mediated behavioral intoxication in a number of species including *Caenorhabditis elegans* (15), and fruit flies (16). BK channels are

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[3] The abbreviations used are: BK, large conductance voltage- and Ca^{2+}-gated potassium channel; hSlo, human *slo-1* 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.

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% CO_2 incubator. Cells were transfected with α BK Ser-869 or S869A constructs (generously provided by Dr. Michael Shipston), alone or in combination with β4-tGFP (Origene) for 48 h using Effectene (Qiagen) according to the manufacturer's instructions. Following transfection, the media was replaced with fresh media and recordings were conducted 3–7 days *in vitro* from the start of transfection.

Immunoprecipitation, SDS-PAGE, and Immunoblot—For immunoprecipitation experiments, 100-mm dishes of transfected HEK 293T cells were serum-starved for 24 h before treatment with 0.1% dH_2O (vehicle control treatment), 250 μM 8-Br-cAMP (Calbiochem) or 250 μM 8-Br-cAMP, and 100 nM-(PKA-(14–22) (Calbiochem) for 10 min. Following treatment, cells were immediately placed on ice, media was removed and cells were washed with 5 ml of cold 1× Hanks' balanced salt solution (with Ca^{2+} and Mg^{2+}, without phenol red) + 10 mM HEPES (Invitrogen). Wash buffer was removed completely, and 800 μl of cold IP buffer (26) with protease inhibitors (Sigma), phosphatase inhibitors (Sigma, mixture inhibitors #3), and 0.2 mM PMSF was added to each dish before mechanically lysing cells with a cell scraper. Cells were further mechanically lysed by repeated aspiration (5 times) through a 20-gauge needle and then spun at 13,400 × *g* in an Eppendorf tabletop microcentrifuge for 10 min at 4 °C to pellet cellular debris. Supernatant was moved to a different tube before measuring the protein concentration using standard Bradford assay conditions. Lysates (200 μg) were pre-cleared with 20 μl of Protein A/G Plus-agarose (Santa Cruz Biotechnology) and 1 μg of rat IgG (Jackson ImmunoResearch) for 30 min at 4 °C. The α BK ZERO HA-tagged channels were immunoprecipitated from the cleared lysate with rat anti-HA affinity purified antibody (Roche Applied Science, 1 μg of antibody pre-bound for 4 h at 4 °C to 40 μl of Protein A/G Plus-agarose) for 12 h at 4 °C. Samples were washed 4 times with 400 μl of cold IP buffer as described above with a 5-min centrifugation at 1,000 × *g* and 4 °C to pellet agarose. After the final wash, IP buffer was completely removed, replaced with 40 μl of 2× loading buffer (8 M urea, 62 mM Tris-HCl, pH 6.8, 20 mM EDTA, 4% SDS, 0.015% bromphenol blue, and 5% 2-mercaptoethanol), and put on ice for 10 min before heating samples at 90 °C for 2 min. Agarose was pelleted and samples were removed to load in parallel with 10 μg of total protein lysates per well on 4–20% Mini-Protean TGX pre-cast gels (Bio-Rad). After dry transfer at constant 25 V for 10 min onto PVDF (Bio-Rad) using the Trans-Blot Turbo system (Bio-Rad), membranes were incubated in Odyssey Blocking Buffer (LiCor) + 1 mM NaF for 1 h at room temperature and then incubated overnight at 4 °C in primary antibodies. Rabbit anti-PKA phospho-substrate specific (1:500, Cell Signaling), rat anti-HA (Roche Applied Science), and mouse anti-GAPDH (1:1000, Invitrogen) primary antibodies were diluted in Odyssey Blocking Buffer with 0.2% Tween 20 and 1 mM NaF. Membranes were washed four times for 5 min each in 1× TBS with 0.1% Tween 20 and 1 mM NaF before applying the appropriate infrared dye-conjugated secondary antibodies for 1 h at room temperature. Goat anti-rabbit IR Dye 800CW, goat anti-rat IR Dye 680RD, and goat anti-mouse IR Dye 680RD secondary antibodies (1:15,000, Li-Cor) were diluted in Odyssey Blocking Buffer with 0.2% Tween 20, 0.01% SDS, and 1 mM NaF. After secondary antibody incubation, membranes were washed four times for 5 min each in 1× TBS with 0.1% Tween 20 and 1 mM NaF before a final rinse in 1× TBS and 1 mM NaF. Membranes were imaged on an Odyssey Classic Infared Imaging System. Band densitometry was done in ImageJ after background subtraction and band intensity for rat anti-HA and rabbit anti-PKA phospho-substrate specific labeled bands was measured. Ratio of the PKA phospho-substrate specific/HA labeling was calculated for each treatment condition and normalized to the control-treated mean for three independent experiments, using GraphPad Prism 6 for statistical comparison (one-way analysis of variance) of different treatment conditions.

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 vibroslicer (Vibratome 3000). Slices were incubated for up to 6 h at room temperature (20–22 °C) in a gassed (95% O_2 and 5% CO_2) $NaHCO_3$-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% O_2 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 PO_4-free Hank's solution (in mM: NaCl 120; KCl 10; $MgCl_2$ 2; $CaCl_2$ 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 hematocrit tubes. Pipette tips were positioned in the "mouth" of the hematocrit 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 K_2MeSO_4, 2 $MgCl_2$, 2 $CaCl_2$, 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 substraction 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 NP_o 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, NP_o—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 (P_o). 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 NP_o 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 NP_o ratios generated for the first exposure used this control for normalization of the data. BK channel activity was measured as NP_o ratio percent ((NP_o/NP_o control) × 100). The calculations of NP_o 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. 1*A* shows representative traces and Fig. 1*B* the averaged open probability of BK channels from three different membrane

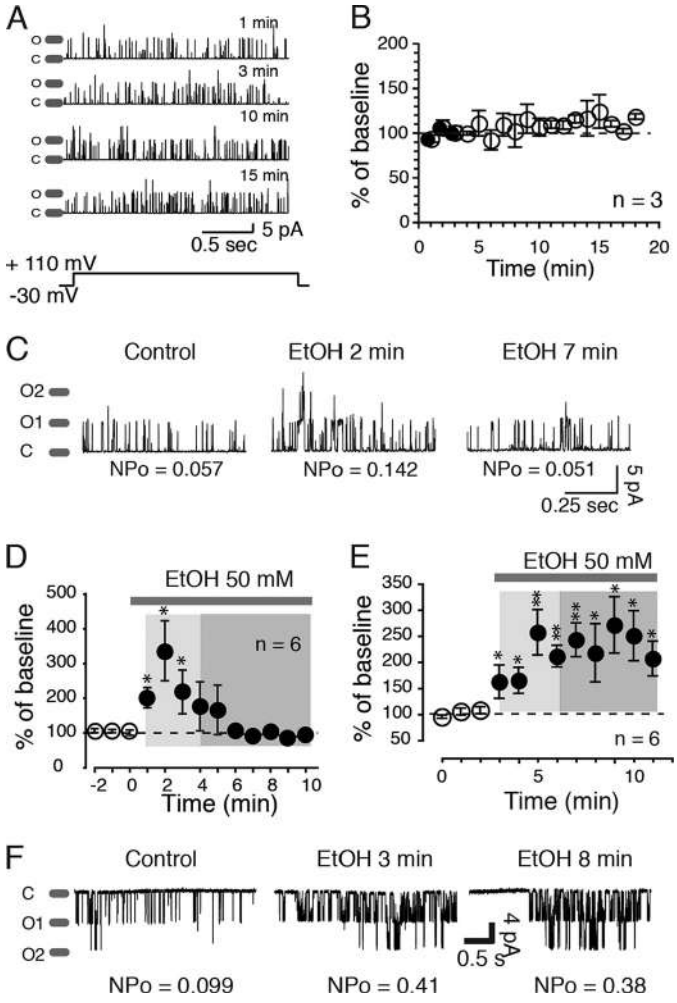

FIGURE 1. **Acute effects of EtOH on α and αβ4 BK channels in HEK 293 cells.** *A*, traces of the same α BK channel at different time points (between 1 and 15 min). Currents were evoked by depolarizing the membrane potential to +110 mV from a holding of −30 mV, as represented by the square pulse, *below 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 value (100%) (first three measurements). *C*, representative traces of a 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 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* traces. *Asterisks* represent statistically significant differences from baseline: * indicate $p \leq 0.05$.

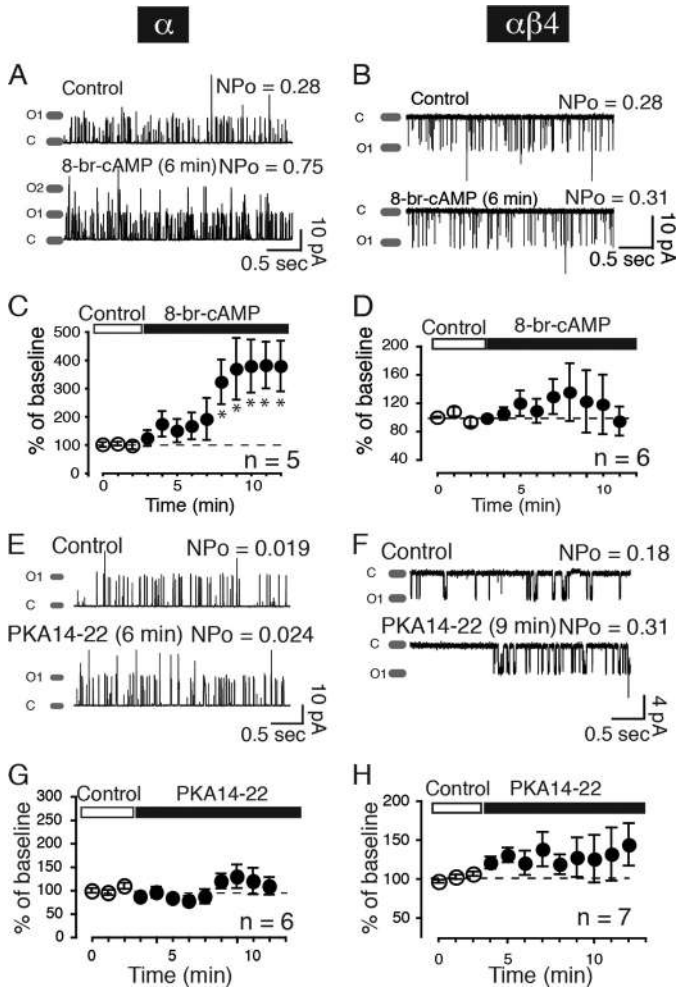

FIGURE 2. **Effect of 8-Br-cAMP and PKA-(14–22) on α and αβ4 BK channels in HEK 293 cells.** *A* and *B*, representative traces of α and αβ4 BK channel activity before (control) and during 8-Br-cAMP exposure (6 min). The *numbers above* the traces indicate open probability. *C* and *D*, averaged BK channel activity before and during 8-Br-cAMP exposure. The *dashed line* shows the baseline control level. *E* and *F*, representative traces of α and αβ4 BK channel activity before (control) and during PKA-(14–22) exposure (6 min). *G* and *H*, averaged BK channel activity before and during exposure to PKA inhibitor, PKA-(14–22). The *dashed line* shows the baseline control level. The open probability is indicated by the *numbers above* the traces. 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: * indicate $p \leq 0.05$.

patches, plotted as a function of time, demonstrating the stability of BK activity.

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. 1*C*). This response was followed by a rapid return of open probability to pre-exposure levels, illustrated with representative data from a single experiment ($NP_o = 0.051$; Fig. 1*C*, *EtOH 7 min*), and as an average from multiple recordings in Fig. 1*D* (*darker gray box*). As with α BK, αβ4 BK channel open probability increased (from 0.10 to 0.41 Fig. 1*F*, *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 NP_o remained elevated in the continued presence of EtOH (Fig. 1*F*, *EtOH 8 min*). When averaged over 6 patches, EtOH potentiated αβ4 BK channel activity by 2.56 ± 0.43-fold (Fig. 1*E*), and this potentiation was undiminished 8 min after alcohol exposure (Fig. 1*E*, *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 activator. Fig. 2*A* shows representative traces

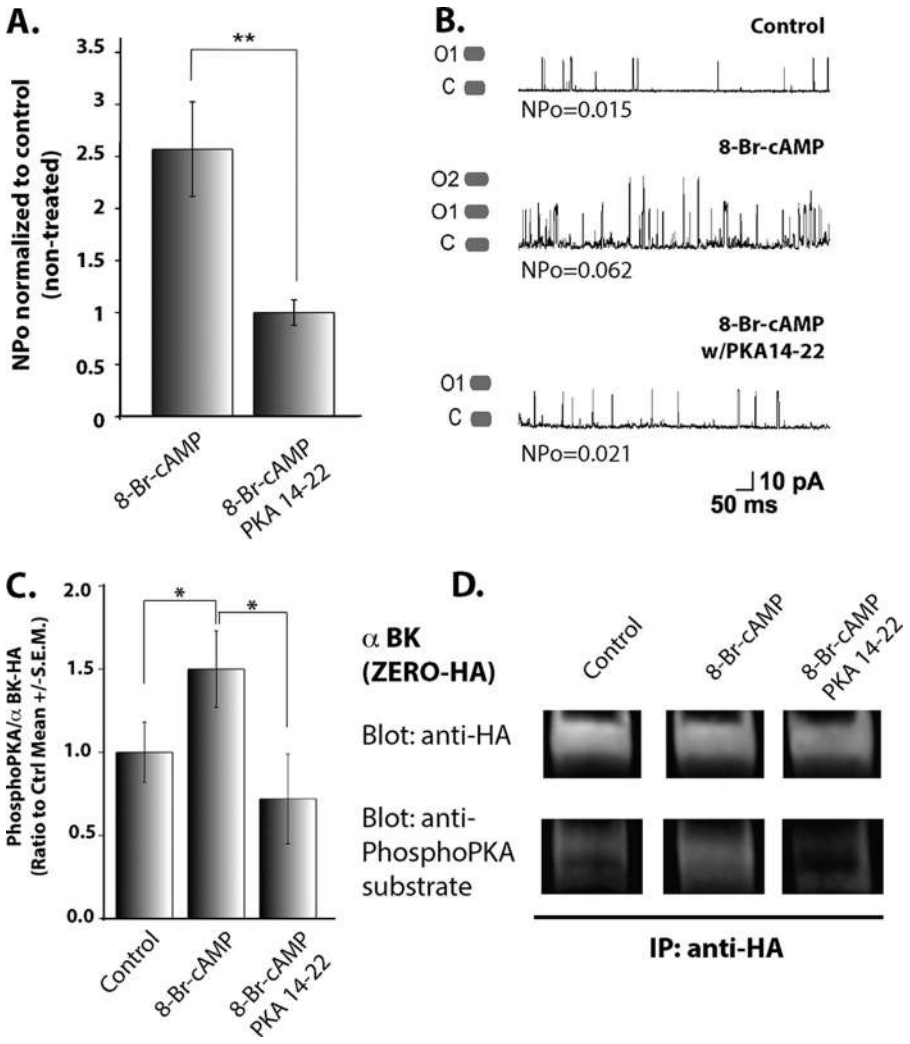

FIGURE 3. **Effect of PKA-(14–22) on 8-Br-cAMP activation and phosphorylation of α BK channels in HEK 293 cells.** *A,* bar graph quantifying NP_o in the presence of 8-Br-cAMP and 8-Br-cAMP combined with PKA-(14–22) normalized to control for each cell during cell-attached patch clamp recordings. *B,* representative traces of α BK channel activity before (control), during 8-Br-cAMP exposure, and during 8-Br-cAMP with PKA-(14–22) exposure monitored averaged every 10 min. The *numbers below* the traces indicate open probability for each representative trace. Number of cells tested for each condition was 4. *C* and *D,* immunoprecipitation of α BK transiently expressed in HEK 293 cells shows direct phosphorylation of the α BK channel complex by PKA. *C,* quantification of phospho-PKA substrate immunoblot intensity of anti-HA IPs following 10-min treatments ($n = 3$ independent experiments). *D,* representative images of α BK and phospho-PKA immunoblots of anti-HA IPs from control, 8-Br-cAMP, and 8-Br-cAMP + PKAi. *Asterisks* represent statistically significant differences: ** indicates $p \leq 0.01$ and * indicates $p \leq 0.05$.

illustrating the increase of α 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. 2*C*). 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. 3*A*). Adding the PKA inhibitor blocked the increase, resulting in an NP_o statistically unchanged from non-treated (0.86 ± 0.1219, $n = 4$) (Fig. 3*A*). Therefore, activation resulting from 8-Br-cAMP is likely dependent upon cAMP-dependent protein kinase activity. Immunoprecipitation of α BK-HA transiently expressed in HEK 293T cells also corroborates direct phosphorylation of the channel complex by PKA. Quantification of normalized phospho-PKA substrate immunoblot intensity of anti-HA IPs indicate an increase from control (1.00 ± 0.18) to 1.50 ± 0.23, after a 10-min treatment with 8-Br-cAMP. Co-incubation of 8-Br-cAMP with PKA-(14–22) blocked the increase in PKA-induced phosphorylation (0.72 ± 0.27) (Fig. 3, *C* and *D*).

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. 2*E* (0.019 before and 0.024 3 min after exposure to PKA-(14–22)), and by the averaged NP_o of BK channels in patches from 6 different cells (Fig. 2*G*). 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-

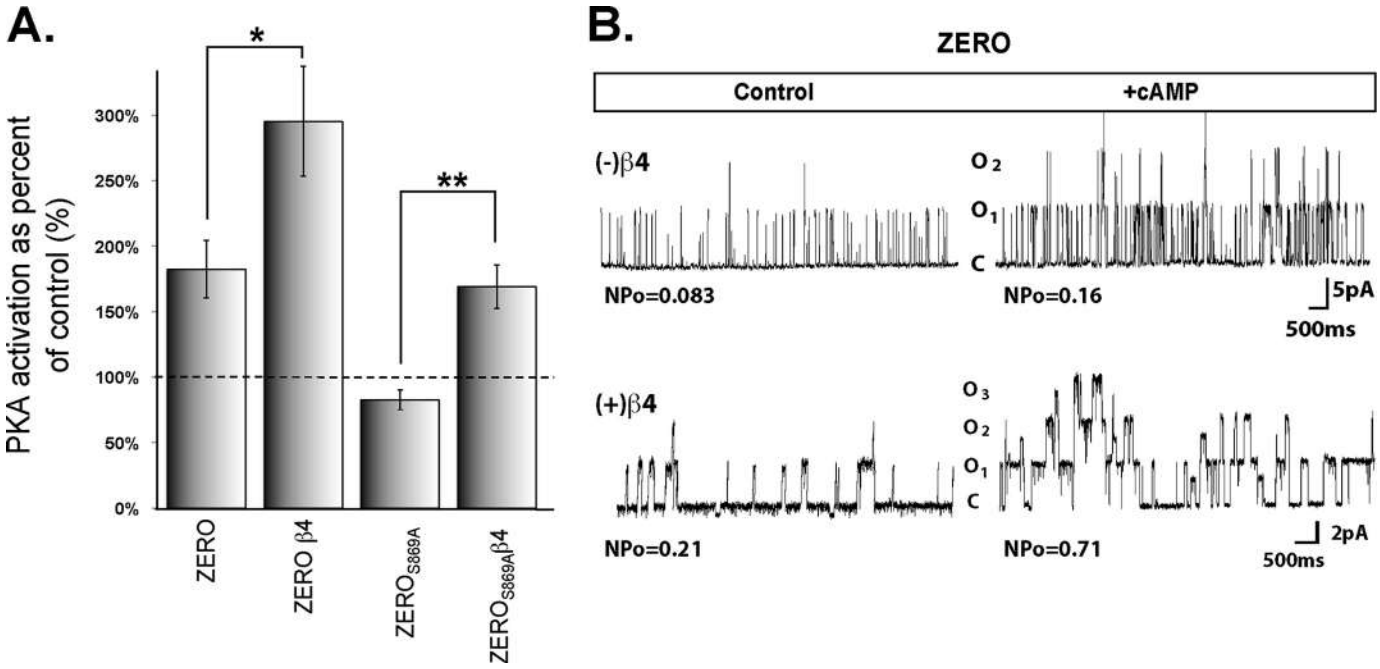

FIGURE 4. **Effect of PKA activation on α and $\alpha\beta4$ BK channel NP_o in the wild-type channel and in channel expressing S869A mutation in HEK 293 recorded using excised patches.** *A,* bar graph quantifying NP_o of individual inside-out patch clamp recordings with 0.1 mM cAMP in the bath solution 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 $\alpha\beta4$ (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: *, $p \leq 0.05$, and **, $p \leq 0.01$.

theless examined the effect of PKA-(14–22) (Fig. 2, *F* and *H*), which did not produce a statistically significant change in $\alpha\beta4$ BK channel open probability.

To explore the mechanism of PKA activation, we assayed the functional regulation of α and $\alpha\beta4$ BK channels via PKA utilizing expression of point-mutated cDNA constructs on a specific PKA regulation site within the α subunit. Tian and colleagues (43), demonstrated that the α 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 α 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 $\beta4$ 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 α 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

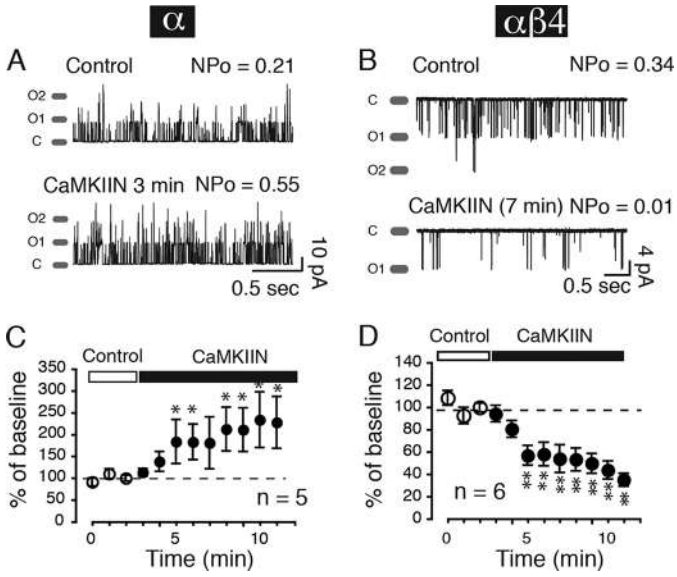

FIGURE 5. **Effects of inhibiting CaMKII on α and $\alpha\beta4$ BK channel open probability.** *A* and *B,* sample traces of BK channel activity before and during drug exposure. The *right-hand side* of each trace shows the NP_o measured during 20 s recording. *C* and *D,* average BK channel activity 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 $p \leq 0.05$, and ** indicates $p \leq 0.01$.

either α and $\alpha\beta4$ 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 $\beta4$ ($\alpha\beta4$ BK) (Fig. 4, *A* and *B*). As previously observed, channel

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 α BK 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. 4*A*). 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 α BK 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 α BK S869A channels (Fig. 5*A*). These data suggest that the β4 subunits either have sites directly phosphorylated by PKA, which result in an additive/synergistic effect with endogenous α BK activation, or the αβ4 association fundamentally changes how PKA interacts with BK channels, possibly favoring PKA phosphorylation sites other than Ser-869 on the α BK 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. CaMKIIN (100 nM), a membrane-permeable selective CaMKII inhibitor markedly increased α BK channel activity. Fig. 5*A* shows representative records of α BK activity, which increased nearly 2.5-fold when compared with pre-treatment (control, 0.21; 3 min post-CaMKIIN, 0.55). The increase, after averaging α BK channel activity from five different membrane patches, is shown in Fig. 5*C*. These data suggest that α BK channels are tonically inhibited by CaMKII. In stark contrast, the αβ4 BK channel was markedly attenuated by CaMKIIN, reduced from an NP_o of 0.34 to 0.01 (Fig. 5*B*). 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. 5*D*). 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*, α BK channel activity increased by more than 3-fold (to $NP_o = 0.095$) 6 min after OA exposure, compared with pre-treatment ($NP_o = 0.03$). This potentiation is visible 1 min after okadaic acid exposure and increases steadily to reach a plateau after 7–8 min (Fig. 6*C*). In contrast, we found that 1 μM okadaic acid markedly attenuated αβ4 BK channel activity. Thus, Fig. 6, *B* and *D*, show that the open probability of this channel (0.28 pre-treatment; Fig. 6*B*, *top trace*) decreased to 0.09, 5 min after okadaic acid superfusion (Fig. 6*B*, *bottom trace*). On average (6 patches), okadaic acid attenuated αβ4 BK activity by about 64% (Fig. 6*D*). Thus, co-expression of the β4 subunit reverses the effects of phosphatase inhibition observed with the α BK subunit alone.

We next examined the influence of β4 on activation of phosphatase activity. Activation of PP2A by 30 μM *N*-hexanoyl D-erythrosphingosine increased αβ4 BK channel activity in 5 cells by about 150% (Fig. 6*F*), whereas application to α BK channels had no statistically significant effect (Fig. 6*E*).

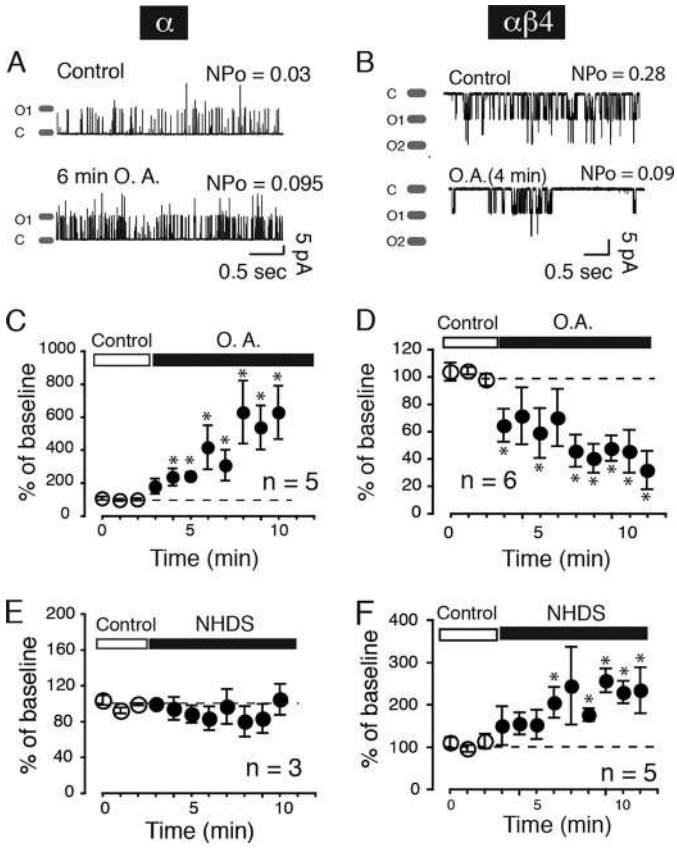

FIGURE 6. **Effects of okadaic acid (*OA*) and protein phosphatase A_2 activator *N*-hexanoyl-D-erythro-sphingosine (*NHDS*) on α and αβ4 BK channel.** *A* and *B*, sample traces of BK channel activity before and during drug exposure. On the *right-hand side* of each trace is the NP_o measured during 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 α BK 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. 1*D*). 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

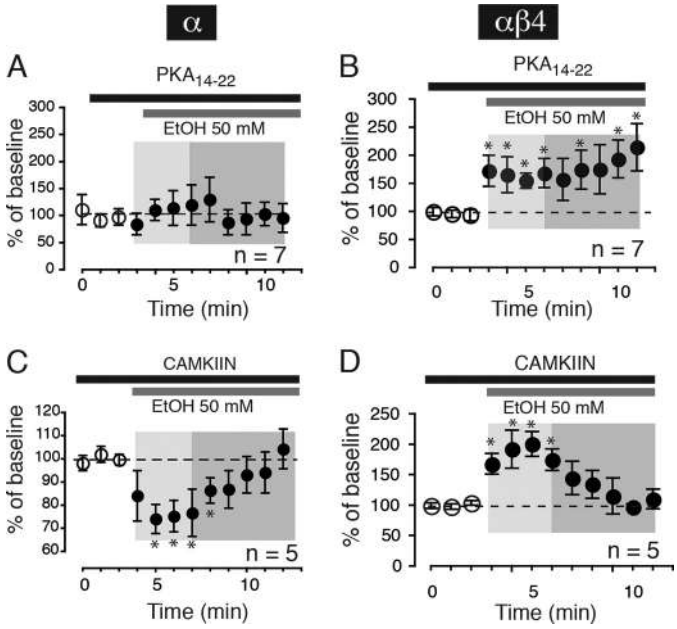

FIGURE 7. **Averaged effects of PKA and CaMKII inhibition on EtOH-treated α and αβ4 BK channels.** The *dark bars* indicate the timing of exposing HEK 293 cells to the blockers. The *lighter bars* show when EtOH was applied. The *lighter* and *darker gray shaded boxes* show where acute potentiation and tolerance, respectively, typically occur. Open probability is expressed as % of (control) baseline. *A* and *B*, averaged effects of PKA-(14–22) on α and αβ4 BK channel. *C* and *D*, averaged effects of CaMKIIN on α and αβ4 BK channel. Number of patches tested in each condition is indicated by the *n* value shown in the *lower part* of the graphs. The *dashed line* shows the baseline control level. *Asterisks* represent statistically significant differences from baseline: * indicates $p \leq 0.05$.

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. 7*A*, *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 100 nM PKA-(14–22) failed to affect the potentiation of the αβ4 BK channel open probability by 50 mM ethanol (Fig. 7*B*), when averaged over 7 patches. Additionally, potentiation was still evident 9 min after the start of alcohol application. The αβ4 BK activity was increased by more than 2-fold compared with control values (Fig.7, *open circles*). These results are in line with what we found under control conditions, without PKA inhibitor in the bath (Fig. 1*E*). Our results strongly suggest that in the presence of the β4 subunit, PKA modulation of the BK channel is no longer necessary for potentiation of the channel by alcohol. The results also indicate that the sustained response of this channel to alcohol is similarly independent of PKA.

Involvement of the CaMKII Pathway in the Response of BK to EtOH—After exposure to 100 nM CaMKIIN, a CaMKII inhibitor, the activity of the α BK channel was markedly inhibited by ethanol (Fig. 7*C*; $n = 5$). This inhibition was short-lived and presented the hallmark of tolerance, as the averaged activity returned to control levels 6–7 min after the beginning of exposure (Fig. 7*C*). Interestingly, incubation with CaMKIIN did not block the initial increase of αβ4 BK channel NP_o (Fig. 7*D*), but it reintroduced the acute tolerance blocked by β4 under control conditions (Fig. 1, *D* and *E*). Thus, 3 min after the beginning of exposure, BK channel activity slowly diminished and returned to control levels 3–4 min later (Fig. 7*D*), suggesting a role for CaMKII-mediated channel phosphorylation in the block of acute tolerance by β4.

Effect of Phosphatases on the BK EtOH Response—Because α BK channel potentiation appeared to involve phosphorylation through activation of PKA, we wondered whether a phosphatase could be responsible for the rapid tolerance that develops within minutes after exposure. We incubated cells with 1 μM okadaic acid prior to recording α BK channel activity. After recording a stable baseline, we applied 50 mM ethanol and found that channel potentiation was sustained over the a 10-min recording period following exposure (data not shown), unlike what we observed in the absence of okadaic acid (Fig. 1*D*). 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. 8*B*). The maximum increase of αβ4 BK channel NP_o (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. 8*C*). However, this increase was not sustained and soon returned to control levels (Fig. 8*C*, *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. 8*D*). 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.

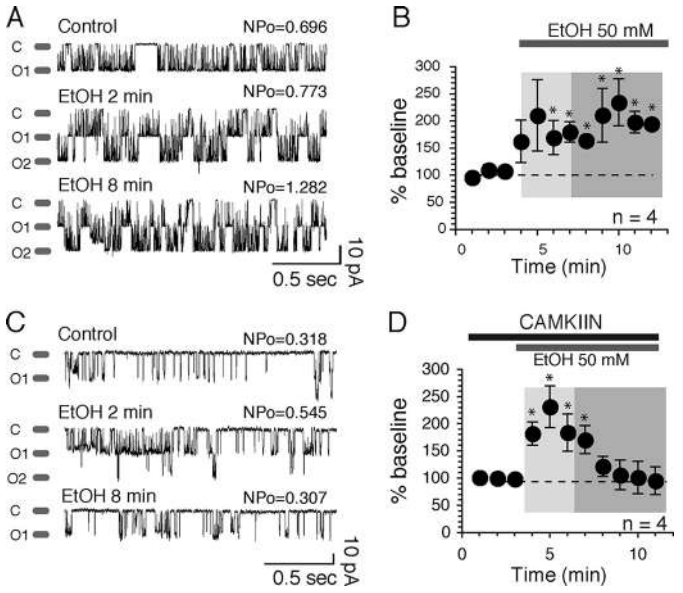

FIGURE 8. **Response of NAcc medium spiny neurons to acute EtOH with and without CaMKII blocker.** *A,* representative traces at $V_h = -60$ mV of presumably αβ4 NAcc BK channel activity before (control) and during EtOH application (EtOH, 2 and 8 min). *B,* graph summarizing the averaged effects of EtOH on BK channel open probability, expressed as % of baseline. The *dashed line* shows the baseline control level. Alcohol application is shown by a *filled horizontal bar above* the graph. *C,* representative traces of BK channels exposed to EtOH in the presence of CaMKII blocker. *D,* averaged effect of EtOH on BK channels from 4 patches. *Lighter* and *darker shaded boxes* indicate BK channel potentiation and tolerance, respectively, in the presence of EtOH. *Asterisks* represent statistically significant differences from baseline: * indicates $p \leq 0.05$.

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 channel complex. 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 in response to PKA, most notably in the absence of CaMKII phosphorylation.

It is important to note that increases in NP_o in response to PKA were *not* observed in αβ4 BK channels recorded in cell-attached configuration. The key difference in their response is likely the absence of CaMKII tonic activation when recording from excised inside-out patches (32). This strongly suggests

TABLE 1

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

Searches were done using the PhosphoMotif Finder (60), which yielded nine PKA kinase and four CaMKII substrate motifs overall. Sites enclosed within a box are predicted to be within the intracellular domains of the β4 subunit. *Below:* the hβ4 (GenBank accession number AF215891) protein sequence used during the search showing transmembrane domains (*boxes*) (61) and predicted intracellular domains (*lines*).

	Position in Protein	Sequence in Protein	Corresponding motif described in the literature (phosphorylated residues in red)	Features of motif described in the literature
1	17 - 19	SIR	[pS/pT]X[R/K]	PKA kinase substrate motif
2	77 - 79	RGT	[R/K]X[pS/pT]	PKA kinase substrate motif
3	77 - 80	RGTS	RXXpS	Calmodulin-dependent protein kinase II substrate motif
4	77 - 80	RGTS	RXXpS	PKA kinase substrate motif
5	77 - 80	RGTS	RXX[pS/pT]	Calmodulin-dependent protein kinase II substrate motif
6	112 - 114	KCS	[R/K]X[pS/pT]	PKA kinase substrate motif
7	125 - 129	KNLES	KXXX[pS/pT]	PKA kinase substrate motif
8	206 - 210	KRKFS	KXXX[pS/pT]	PKA kinase substrate motif
9	207 - 210	RKFS	RXXpS	Calmodulin-dependent protein kinase II substrate motif
10	207 - 210	RKFS	RXXpS	PKA kinase substrate motif
11	207 - 210	RKFS	RXX[pS/pT]	Calmodulin-dependent protein kinase II substrate motif
12	207 - 210	RKFS	[R/K][R/K]X[pS/pT]	PKA kinase substrate motif
13	208 - 210	KFS	[R/K]X[pS/pT]	PKA kinase substrate motif

1 MAKLRVAYEY TEAEDKSIRL GLFLIISGVV SLFIFGFCWL SPALQDLQAT

51 HSDEHQLLTN PKCSYIPPCK RENQKNLESV MNWQQYWKDE IGSQPFTCYF

101 EANCTVLSVQ QIGEVFECTF TCGADCRGTS QYPCVQVYVN NSESNSRALL

151 NQHQRPDDVL LHRTHDEIVL LHCFLWPLVT FVVGVLIVVL TICAKSLAVK

201 AEAMKKRKFS

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. 4*A*) (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

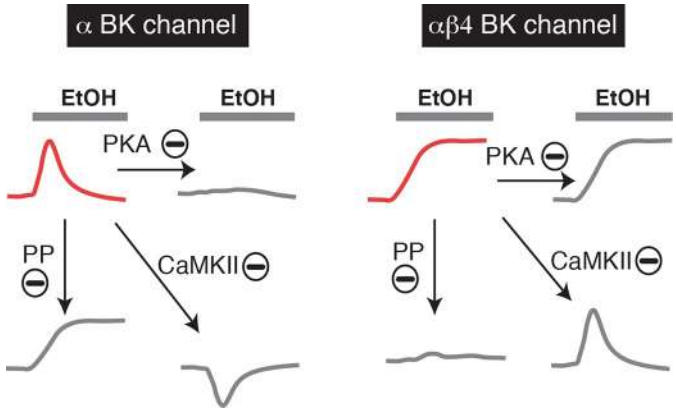

FIGURE 9. **Schematic of the role of kinases and phosphatases in mediating the effects of alcohol on α and αβ4 BK channels activity.** The *thick red lines* represent the temporal response of α and αβ4 BK channels to acute EtOH, shown in Fig. 1 in the absence of kinase/phosphatase blockers. α BK channels are initially potentiated before developing rapid tolerance (*left panel*). In α subunits, inhibition of PKA totally blocks the channel response to EtOH, whereas CaMKII antagonist induces the channels to be transiently inhibited. In the presence of a phosphatase inhibitor, α BK channel tolerance disappears entirely. In contrast, tolerance is not observed when α is coexpressed with the β4 subunit (*red line*, *right panel*), but appears when BK channels are treated with a CaMKII inhibitor. Unlike with α subunit alone, inhibition of PKA has no effect on the modulation of EtOH of channel activity, whereas blockade of phosphatases leads to a transitory dip of channel activity. Taken together, this schematic highlights how β4 subunit expression transforms the role of PKA and CaMKII in mediating EtOH effects on BK channels.

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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REFERENCES

1. Carroll, M. E., Anderson, M. M., and Morgan, A. D. (2007) Higher locomotor response to cocaine in female (vs. male) rats selectively bred for high (HiS) and low (LoS) saccharin intake. *Pharmacol. Biochem. Behav.* **88,** 94–104
2. Simms, J. A., Steensland, P., Medina, B., Abernathy, K. E., Chandler, L. J., Wise, R., and Bartlett, S. E. (2008) Intermittent access to 20% ethanol induces high ethanol consumption in Long-Evans and Wistar rats. *Alcohol Clin. Exp. Res.* **32,** 1816–1823
3. Mutschler, N. H., Covington, H. E., 3rd, and Miczek, K. A. (2001) Repeated self-administered cocaine "binges" in rats: effects on cocaine intake and withdrawal. *Psychopharmacology* **154,** 292–300
4. Melendez, R. I. (2011) Intermittent (every-other-day) drinking induces rapid escalation of ethanol intake and preference in adolescent and adult C57BL/6J mice. *Alcohol Clin. Exp. Res.* **35,** 652–658
5. Cicero, T. J., Bernard, J. D., and Newman, K. (1980) Effects of castration and chronic morphine administration on liver alcohol dehydrogenase and the metabolism of ethanol in the male Sprague-Dawley rat. *J. Pharmacol. Exp. Ther.* **215,** 317–324
6. Erwin, V. G., McClearn, G. E., and Kuse, A. R. (1980) Interrelationships of alcohol consumption, actions of alcohol, and biochemical traits. *Pharmacol. Biochem. Behav.* **13,** 297–302
7. Schuckit, M. A., Smith, T. L., Hesselbrock, V., Bucholz, K. K., Bierut, L., Edenberg, H., Kramer, J., Longacre, E., Fukukura, T., Kalmijn, J., Danko, G. P., and Trim, R. (2008) Clinical implications of tolerance to alcohol in nondependent young drinkers. *Am. J. Drug Alcohol Abuse* **34,** 133–149
8. Schuckit, M. A., (1994) Low level of response to alcohol as a predictor of future alcoholism. *Am. J. Psychiatry* **151,** 184–189
9. Yuan, C., O'Connell, R. J., Wilson, A., Pietrzykowski, A. Z., and Treistman, S. N. (2008) Acute alcohol tolerance is intrinsic to the BKCa protein, but is modulated by the lipid environment. *J. Biol. Chem.* **283,** 5090–5098
10. Treistman, S. N., and Martin, G. E. (2009) BK channels: mediators and models for alcohol tolerance. *Trends Neurosci.* **32,** 629–637
11. Martin, G. E., Hendrickson, L. M., Penta, K. L., Friesen, R. M., Pietrzykowski, A. Z., Tapper, A. R., and Treistman, S. N. (2008) Identification of a BK channel auxiliary protein controlling molecular and behavioral tolerance to alcohol. *Proc. Natl. Acad. Sci. U.S.A.* **105,** 17543–17548
12. Yan, J., and Aldrich, R. W. (2012) BK potassium channel modulation by leucine-rich repeat-containing proteins. *Proc. Natl. Acad. Sci. U.S.A.* **109,** 7917–7922
13. Behrens, R., Nolting, A., Reimann, F., Schwarz, M., Waldschütz, R., and Pongs, O. (2000) hKCNMB3 and hKCNMB4, cloning and characterization of two members of the large-conductance calcium-activated potassium channel beta subunit family. *FEBS Lett.* **474,** 99–106
14. Brenner, R., Jegla, T. J., Wickenden, A., Liu, Y., and Aldrich, R. W. (2000) Cloning and functional characterization of novel large conductance calcium-activated potassium channel β subunits, hKCNMB3 and hKCNMB4. *J. Biol. Chem.* **275,** 6453–6461
15. Davies, A. G., Pierce-Shimomura, J. T., Kim, H., VanHoven, M. K., Thiele, T. R., Bonci, A., Bargmann, C. I., and McIntire, S. L. (2003) A central role of the BK potassium channel in behavioral responses to ethanol in *C. elegans*. *Cell* **115,** 655–666
16. Cowmeadow, R. B., Krishnan, H. R., and Atkinson, N. S. (2005) The slowpoke gene is necessary for rapid ethanol tolerance in *Drosophila*. *Alcohol Clin. Exp. Res.* **29,** 1777–1786
17. Crowley, J. J., Treistman, S. N., and Dopico, A. M. (2003) Cholesterol antagonizes ethanol potentiation of human brain BKCa channels reconstituted into phospholipid bilayers. *Mol. Pharmacol.* **64,** 365–372
18. Dopico, A. M., Lemos, J. R., and Treistman, S. N. (1996) Ethanol increases the activity of large conductance, Ca^{2+}-activated K^+ channels in isolated neurohypophysial terminals. *Mol. Pharmacol.,* **49,** 40–48
19. Knott, T. K., Dopico, A. M., Dayanithi, G., Lemos, J., and Treistman, S. N. (2002) Integrated channel plasticity contributes to alcohol tolerance in neurohypophysial terminals. *Mol. Pharmacol.* **62,** 135–142
20. Martin, G., Puig, S., Pietrzykowski, A., Zadek, P., Emery, P., and Treistman, S. (2004) Somatic localization of a specific large-conductance calcium-activated potassium channel subtype controls compartmentalized ethanol sensitivity in the nucleus accumbens. *J. Neurosci.* **24,** 6563–6572
21. Koob, G. F. (1998) Drug abuse and alcoholism: overview. *Adv. Pharmacol.* **42,** 969–977
22. Erxleben, C., Everhart, A. L., Romeo, C., Florance, H., Bauer, M. B., Alcorta, D. A., Rossie, S., Shipston, M. J., and Armstrong, D. L. (2002) Interacting effects of N-terminal variation and strex exon splicing on slo potassium channel regulation by calcium, phosphorylation, and oxidation. *J. Biol. Chem.* **277,** 27045–27052
23. Fukao, M., Mason, H. S., Britton, F. C., Kenyon, J. L., Horowitz, B., and Keef, K. D. (1999) Cyclic GMP-dependent protein kinase activates cloned BKCa channels expressed in mammalian cells by direct phosphorylation at serine 1072. *J. Biol. Chem.* **274,** 10927–10935
24. Nara, M., Dhulipala, P. D., Wang, Y. X., and Kotlikoff, M. I. (1998) Reconstitution of β-adrenergic modulation of large conductance, calcium-activated potassium (maxi-K) channels in *Xenopus* oocytes: identification of the cAMP-dependent protein kinase phosphorylation site. *J. Biol. Chem.* **273,** 14920–14924
25. Tian, B., and Mathews, M. B. (2001) Functional characterization of and cooperation between the double-stranded RNA-binding motifs of the protein kinase PKR. *J. Biol. Chem.* **276,** 9936–9944
26. Tian, L., Coghill, L. S., MacDonald, S. H., Armstrong, D. L., and Shipston, M. J. (2003) Leucine zipper domain targets cAMP-dependent protein kinase to mammalian BK channels. *J. Biol. Chem.* **278,** 8669–8677
27. Tian, L., Coghill, L. S., McClafferty, H., MacDonald, S. H., Antoni, F. A., Ruth, P., Knaus, H. G., and Shipston, M. J. (2004) Distinct stoichiometry of BKCa channel tetramer phosphorylation specifies channel activation and inhibition by cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* **101,** 11897–11902
28. Alioua, A., Tanaka, Y., Wallner, M., Hofmann, F., Ruth, P., Meera, P., and Toro, L. (1998) The large conductance, voltage-dependent, and calcium-sensitive K^+ channel, hSlo, is a target of cGMP-dependent protein kinase phosphorylation *in vivo*. *J. Biol. Chem.* **273,** 32950–32956
29. Swayze, R. D., and Braun, A. P. (2001) A catalytically inactive mutant of type I cGMP-dependent protein kinase prevents enhancement of large conductance, calcium-sensitive K^+ channels by sodium nitroprusside and cGMP. *J. Biol. Chem.* **276,** 19729–19737
30. Alioua, A., Mahajan, A., Nishimaru, K., Zarei, M. M., Stefani, E., and Toro, L. (2002) Coupling of c-Src to large conductance voltage- and Ca^{2+}-activated K^+ channels as a new mechanism of agonist-induced vasoconstriction. *Proc. Natl. Acad. Sci. U.S.A.* **99,** 14560–14565
31. Ling, S., Woronuk, G., Sy, L., Lev, S., and Braun, A. P. (2000) Enhanced activity of a large conductance, calcium-sensitive K^+ channel in the presence of Src tyrosine kinase. *J. Biol. Chem.* **275,** 30683–30689
32. van Welie, I., and du Lac, S. (2011) Bidirectional control of BK channel open probability by cAMKII and PKC in medial vestibular nucleus neurons. *J. Neurophysiol.* **105,** 1651–1659
33. Liu, J., Asuncion-Chin, M., Liu, P., and Dopico, A. M. (2006) CaM kinase II phosphorylation of slo Thr107 regulates activity and ethanol responses of BK channels. *Nat. Neurosci.* **9,** 41–49
34. Wang, Z. W. (2008) Regulation of synaptic transmission by presynaptic CaMKII and BK channels. *Mol. Neurobiol.* **38,** 153–166
35. Gordon, A. G. (1993) Alcohol, deafness, epilepsy, and autism. *Alcohol Clin. Exp. Res.* **17,** 926–928
36. Nagy, L. E. (1996) Alcohol and tyrosine kinase/phosphatase mediated processes. *Alcohol Clin. Exp. Res.* **20,** 106A-108A

37. Wang, Z. J., and Wang, L. X. (2006) Phosphorylation: a molecular switch in opioid tolerance. *Life Sci.* **79,** 1681–1691
38. Feinberg-Zadek, P. L., and Treistman, S. N. (2007) β-Subunits are important modulators of the acute response to alcohol in human BK channels. *Alcohol Clin. Exp. Res.* **31,** 737–744
39. Ahring, P. K., Strøbaek, D., Christophersen, P., Olesen, S. P., and Johansen, T. E. (1997) Stable expression of the human large-conductance Ca^{2+}-activated K^+ channel α- and β-subunits in HEK293 cells. *FEBS Lett.* **415,** 67–70
40. Tseng-Crank, J., Godinot, N., Johansen, T. E., Ahring, P. K., Strøbaek, D., Mertz, R., Foster, C. D., Olesen, S. P., and Reinhart, P. H. (1996) Cloning, expression, and distribution of a Ca^{2+}-activated K_+ channel β-subunit from human brain. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 9200–9205
41. Martin, G., and Siggins, G. R. (2002) Electrophysiological evidence for expression of glycine receptors in freshly isolated neurons from nucleus accumbens. *J. Pharmacol. Exp. Ther.* **302,** 1135–1145
42. Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* **391,** 85–100
43. Tian, L., Duncan, R. R., Hammond, M. S., Coghill, L. S., Wen, H., Rusinova, R., Clark, A. G., Levitan, I. B., and Shipston, M. J. (2001) Alternative splicing switches potassium channel sensitivity to protein phosphorylation. *J. Biol. Chem.* **276,** 7717–7720
44. Tian, L., Hammond, M. S., Florance, H., Antoni, F. A., Shipston, M. J. (2001) Alternative splicing determines sensitivity of murine calcium-activated potassium channels to glucocorticoids. *J. Physiol.* **537,** 57–68
45. Lippiat, J. D., Standen, N. B., Harrow, I. D., Phillips, S. C., and Davies, N. W. (2003) Properties of BK(Ca) channels formed by bicistronic expression of hSlo α- and β1–4 subunits in HEK293 cells. *J. Membr. Biol.* **192,** 141–148
46. Meera, P., Wallner, M., and Toro, L. (2000) A neuronal beta subunit (KCNMB4) makes the large conductance, voltage- and Ca^{2+}-activated K^+ channel resistant to charybdotoxin and iberiotoxin. *Proc. Natl. Acad. Sci. U.S.A.,* **97,** 5562–5567
47. Kume, H., Takai, A., Tokuno, H., and Tomita, T. (1989) Regulation of Ca^{2+}-dependent K^+-channel activity in tracheal myocytes by phosphorylation. *Nature* **341,** 152–154
48. Carl, A., Kenyon, J. L., Uemura, D., Fusetani, N., and Sanders, K. M. (1991) Regulation of Ca^{2+}-activated K^+ channels by protein kinase A and phosphatase inhibitors. *Am. J. Physiol. Cell Physiol.* **261,** 387–392
49. Meera, P., Anwer, K., Monga, M., Oberti, C., Stefani, E., oro, L., and Sanborn, B. M. (1995) Relaxin stimulates myometrial calcium-activated potassium channel activity via protein kinase A. *Am. J. Physiol. Cell Physiol.* **269,** C312–C317
50. Dworetzky, S. I., Boissard, C. G., Lum-Ragan, J. T., McKay, M. C., Post-Munson, D. J., Trojnacki, J. T., Chang, C. P., and Gribkoff, V. K. (1996) Phenotypic alteration of a human BK (hSlo) channel by hSloβ subunit coexpression: changes in blocker sensitivity, activation/relaxation and inactivation kinetics, and protein kinase A modulation. *J. Neurosci.* **16,** 4543–4550
51. White, R. E., Lee, A. B., Shcherbatko, A. D., Lincoln, T. M., Schonbrunn, A., and Armstrong, D. L. (1993) Potassium channel stimulation by natriuretic peptides through cGMP-dependent dephosphorylation. *Nature* **361,** 263–266
52. White, R. E., Schonbrunn, A., and Armstrong, D. L. (1991) Somatostatin stimulates Ca^{2+}-activated K^+ channels through protein dephosphorylation. *Nature* **351,** 570–573
53. Chen, L., Tian, L., MacDonald, S. H., McClafferty, H., Hammond, M. S., Huibant, J. M., Ruth, P., Knaus, H. G., and Shipston, M. J. (2005) Functionally diverse complement of large conductance calcium- and voltage-activated potassium channel (BK) α-subunits generated from a single site of splicing. *J. Biol. Chem.* **280,** 33599–33609
54. Sansom, S. C., Ma, R., Carmines, P. K., and Hall, D. A. (2000) Regulation of Ca^{2+}-activated K^+ channels by multifunctional Ca^{2+}/calmodulin-dependent protein kinase. *Am. J. Physiol. Renal Physiol.* **279,** 283–288
55. Jin, P., Weiger, T. M., Wu, Y., and Levitan, I. B. (2002) Phosphorylation-dependent functional coupling of hSlo calcium-dependent potassium channel and its hβ4 subunit. *J. Biol. Chem.* **277,** 10014–10020
56. Hagen, B. M., Bayguinov, O., and Sanders, K. M. (2003) β1-Subunits are required for regulation of coupling between Ca^{2+} transients and Ca^{2+}-activated K^+ (BK) channels by protein kinase C. *Am. J. Physiol. Cell Physiol.* **285,** C1270–C1280
57. Pietrzykowski, A. Z., Friesen, R. M., Martin, G. E., Puig, S. I., Nowak, C. L., Wynne, P. M., Siegelmann, H. T., and Treistman, S. N. (2008) Posttranscriptional regulation of BK channel splice variant stability by miR-9 underlies neuroadaptation to alcohol. *Neuron* **59,** 274–287
58. Yuan, C., O'Connell, R. J., Jacob, R. F., Mason, R. P., and Treistman, S. N. (2007) Regulation of the gating of BKCa channel by lipid bilayer thickness. *J. Biol. Chem.* **282,** 7276–7286
59. Yuan, C., Chen, M., Covey, D. F., Johnston, L. J., and Treistman, S. N. (2011) Cholesterol tuning of BK ethanol response is enantioselective, and is a function of accompanying lipids. *PLoS One* **6,** e27572
60. Amanchy, R., Kandasamy, K., Mathivanan, S., Periaswamy, B., Reddy, R., Yoon, W. H., Joore, J., Beer, M. A., Cope, L., and Pandey, A. (2011) Identification of novel phosphorylation motifs through an integrative computational and experimental analysis of the human phosphoproteome. *J. Proteomics Bioinform.* **4,** 22–35
61. Weiger, T. M., Holmqvist, M. H., Levitan, I. B., Clark, F. T., Sprague, S., Huang, W. J., Ge, P., Wang, C., Lawson, D., Jurman, M. E., Glucksmann, M. A., Silos-Santiago, I., DiStefano, P. S., and Curtis, R. (2000) A novel nervous system beta subunit that down-regulates human large conductance calcium-dependent potassium channels. *J. Neurosci.* **20,** 3563–3570

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