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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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**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 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)\(^5\) 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 \(\beta 4\) subunit has been “knocked out” (KO) suggests remarkable parallels in the effects of the \(\beta 4\) subunit on acute alcohol tolerance at the levels of single channel recording, spike pattern, and behavior (11). At each level, acute tolerance was apparent within a few minutes in \(\beta 4\) KO mice, but not wild-type (WT) mice. Moreover, the \(\beta 4\) 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 \(\alpha\) subunit, which is the product of a single gene, and four regulatory \(\beta\) subunits, products of four distinct genes. Although the \(\alpha\) BK subunit is ubiquitously expressed in the brain (12), only \(\beta 1\) and \(\beta 4\), 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 \(\alpha\)-1 gene that encodes the pore-forming subunit of a large conductance Ca\(^{2+}\)-activated K\(^+\) channel; CaMKII, Ca\(^{2+}\) and calmodulin-dependent protein kinase II; NAcc, nucleus accumbens; 8-Br-cAMP, 8-bromo-cAMP; IP, immunoprecipitation.
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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 Src (30, 31), and CaMKII (32–34) regulate spectrum of kinases, including PKA (22–27), PKG (23, 28, 29), protein kinase C (PKC), cyclic AMP-dependent protein kinase (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).

For 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% dH2O (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 Ca2+ and Mg2+, 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 Infrared 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 vibrissocer (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 chamber. 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 main superfusing solution minutes before recordings to ensure that only channels containing the b4 subunit were recorded.

**Charybdotoxin Treatment and αb4 BK Channels**—Because hSlo αb4 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 exposed to inhibition of the β4 subunit were recorded.

**Electrophysiological Recordings**—We used both the standard single-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 subtraction 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.

**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 NPo 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 αb4 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
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**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 (NP$_{o}$ = 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 NP$_{o}$ 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 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 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 ≤ 0.05.

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**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 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 ≤ 0.05.

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**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 ≤ 0.05.
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 NFp, 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-HA 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 + PKA shows an increase from control (1.00 ± 0.18) to 1.50 ± 0.23, after a 10-min treatment with 8-Br-cAMP (Fig. 3C 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. 2E (0.019 before and 0.024 3 min after exposure to PKA-(14–22)), and by the averaged NFp, 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-

**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, 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 + PKA. Asterisks represent statistically significant differences: ** indicates p < 0.01 and * indicates p < 0.05.
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 β4 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 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

![FIGURE 4. Effect of PKA activation on α and αβ4 BK channel NPo in the wild-type channel and in channel expressing S869A mutation in HEK 293 recorded using excised patches. A, bar graph quantifying NPo 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.](image)

![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 NPo 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.](image)
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. 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 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. 5A). 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. CaMKII (100 nM), a membrane-permeable selective CaMKII inhibitor markedly increased α BK channel activity. Fig. 5A shows representative records of α BK 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 α BK channel activity from five different membrane patches, is shown in Fig. 5C. These data suggest that α BK channels are tonically inhibited by CaMKII. In stark contrast, the αβ4 BK channel was markedly attenuated by CaMKII, reduced from an NPo 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, α BK channel activity increased by more than 3-fold (to NPo = 0.095) 6 min after OA exposure, compared with pre-treatment (NPo = 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 αβ4 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 αβ4 BK activity by about 64% (Fig. 6D). 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 the modulation of phosphorylation activity. Activation of PP2A by 30 μM N-hexanoyl d-erythro-sphingosine increased αβ4 BK channel activity in 5 cells by about 150% (Fig. 6F), whereas application to α BK channels had no statistically significant effect (Fig. 6E).

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. 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...
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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 Ethanol—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 100 nM PKA-(14–22) failed to affect the potentiation of the αβ4 BK channel open probability by 50 mM ethanol (Fig. 7B), 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. 7A, open circles). These results are in line with what we found under control conditions, without PKA inhibitor in the bath (Fig. 1E). 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 Ethanol—After exposure to 100 nM CaMKII, a CaMKII inhibitor, the activity of the α BK channel was markedly inhibited by ethanol (Fig. 7C; 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. 7C). Interestingly, incubation with CaMKII did not block the initial increase of αβ4 BK channel Np (Fig. 7D), 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. 7D), suggesting a role for CaMKII-mediated channel phosphorylation in the block of acute tolerance by β4.

Effect of Phosphatases on the BK Ethanol 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. 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 mM) 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.
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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 the 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 β4 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. 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 α subunit, most notably in the absence of CaMKII phosphorylation.

It is important to note that increases in NP, 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

**DISCUSSION**

**TABLE 1**

<table>
<thead>
<tr>
<th>Position in Protein</th>
<th>Sequence in Protein</th>
<th>Corresponding motif described in the literature</th>
<th>Features of motif described in the literature</th>
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<tbody>
<tr>
<td>1</td>
<td>SR</td>
<td>PKA kinase submotif</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>RGST</td>
<td>PKA kinase submotif</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>RGTS</td>
<td>Calmodulin-dependent protein kinase submotif</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>RGTS</td>
<td>PKA kinase submotif</td>
<td></td>
</tr>
<tr>
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<td>RGTS</td>
<td>Calmodulin-dependent protein kinase submotif</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>12-114 KE5</td>
<td>PKA kinase submotif</td>
<td></td>
</tr>
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<td>13</td>
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**FIGURE 8. Response of NAcc medium spiny neurons to acute EtOH with and without CaMKII blocker.** A, representative traces at Vh = −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 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 < 0.05.
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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