The BK channel accessory β₁ subunit determines alcohol-induced cerebrovascular constriction

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Abstract

Ethanol-induced inhibition of myocyte BK current causes cerebrovascular constriction, yet the molecular targets mediating ethanol action remain unknown. Using BK channel-forming (cbv1) subunits from cerebral artery myocytes, we demonstrate that ethanol potentiates and inhibits current at Ca^{2+}_i lower and higher than ~15 μM, respectively. By increasing cbv1's apparent Ca^{2+}_i-sensitivity, accessory BK β_1 subunits shift the activation-to-inhibition crossover of ethanol action to <3 μM Ca^{2+}_i, with consequent inhibition of current under conditions found during myocyte contraction. Knocking-down *KCNMB1* suppresses ethanol-reduction of arterial myocyte BK current and vessel diameter. Therefore, BK β_1 is the molecular effector of alcohol-induced BK current inhibition and cerebrovascular constriction.

Keywords

channel auxiliary subunits; KCNMB1; maxiK channel; cerebral artery; alcohol; vasoconstriction

Ethanol at concentrations obtained in circulation during heavy episodic alcohol intake, such as in binge drinking, constricts cerebral arteries in many species, including humans, resulting in reduced brain blood flow [1-4]. Using a rodent model, we demonstrated that alcohol-induced cerebrovascular constriction is linked to ethanol-induced reduction of Spontaneous Transient Outward Currents (STOCs) in the cerebral artery myocyte [2]. In these cells, STOCs result from activation of large conductance, voltage- and Ca^{2+}-gated potassium (BK) channels [5-6]. Notably, acute exposure to clinically-relevant ethanol concentrations (50 mM) may depress BK channel activity [2]. However, the molecular targets and mechanisms determining alcohol inhibition of arterial smooth muscle BK current and, eventually, reduced arterial diameter, remain unknown.

BK channels result from the association of four identical subunits encoded by the *Slo1* (*KCNMA1*) gene [7]. In most tissues, however, slo1 subunits interact with a variety of membrane-bound proteins, including small, accessory $\beta_{(1-4)}$ subunits (encoded by four genes, *KCNMB1-4*) that modify BK current phenotype [8-9]. Remarkably, *KCNMB1-4* are differentially expressed across tissues, β_1 being very abundant in vascular smooth muscle yet scarce in other tissues [9]. Taking advantage of our cloning of a BK channel-forming slo1 isoform that is particularly abundant in rat cerebrovascular myocytes ("cbv1", AY330293 [10]) and its associated β_1 subunit (FJ154955), complemented with the *KCNMB1* K/O mouse model, we set to address the specific role of BK channel subunits in alcohol actions on smooth

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muscle BK channel function and its impact on the diameter of resistance-size, small cerebral arteries that control brain blood flow.

Materials and Methods

Cbv1 and β_1 cloning

Rat cerebral artery myocyte isolation and total RNA purification were performed as described [10]. The cloning and functional characterization of cbv1 is provided elsewhere [10-11]. BK β_1 was cloned by PCR using the primers: forward, GCC ATG GGG AAG AAG CTG GTG ATG; reverse, CTA CTT CTG AGC TGC CAA GAC AGA GAG. After initialization at 95° C for 2 min, 30 cycles (94°C for 30 sec, 58°C for 30 sec, 72°C for 1 min) were completed, followed by final elongation at 72°C for 5 min. Amplified PCR products were purified with ultrafree-DA (Millipore) and ligated into PCR-TOPO (Invitrogen). After sequencing 16 clones, the most prevalent BK β_1 isoform from arterial myocytes was ligated into pOX (Aguan Wei; Washington University). Correct insertion and ligation were verified by sequencing at UTennessee HSC Molecular Rsch. Ctr. pOX-β_1 cDNA was linearized with *NotI* and transcribed *in vitro* using T3 (mMessage-mMachine; Ambion).

cRNA injection into *Xenopus* oocytes

Oocytes were removed from *Xenopus laevis* (NASCO) and prepared as described elsewhere [12]. cRNA was dissolved in diethyl polycarbonate-treated water at 5 (cbv1) and 15 (β_1) ng/ μl; 1 μl aliquots were stored at −70°C. Cbv1 (2.5 ng/μl) and β_1 (7.5 ng/μl) cRNAs were coinjected, giving molar ratios ≥6:1 (β:α). Cytosolic cRNA injection (23 nl/oocyte) was conducted using a modified micropipette (Drummond). The interval between injection and patch-clamp recordings was 48-72 h.

Mouse cerebrovascular myocyte isolation

Mice were decapitated using sharp scissors following a procedure approved by the Institutional Animal Care and Use Committee from The University of Tennessee Health Science Center, an AAALAC-accredited institution. Basilar and middle cerebral arteries were dissected out from each brain under a stereozoom microscope (Nikon) and placed in ice-cold dissociation medium (DM) (mM): 0.16 $CaCl_2$, 0.49 EDTA, 10 HEPES, 5 KCl, 0.5 KH_2PO_4, 2 $MgCl_2$, 110 NaCl, 0.5 NaH_2PO_4, 10 $NaHCO_3$, 0.02 phenol red, 10 taurine, 10 glucose. Each artery was cut into 1-2 mm long rings (~30 rings/preparation). Rings were put in 3 ml DM containing 0.00075% papain, 0.05% bovine serum albumin (BSA) and 0.004% dithiothreitol (DTT) for 12 min at 37 °C in a shaking water bath at 60 oscillations/min. Then the incubation solution was replaced with the 3 ml DM that contained 0.06% soybean trypsin inhibitor (STI), 0.05% BSA and 2% collagenase (26.6 units/ml). Incubation was performed for 10 min at the same conditions as before. Finally, the tissue was transferred into 3 ml DM with 0.075% STI and pipetted using a series of borosilicate Pasteur pipettes having fire-polished, diminishing internal diameter tips. BSA was added to the preparation, reaching a final concentration of 0.0625%. The procedure rendered a cell suspension containing relaxed, individual myocytes (≈2 myocytes/field using a 40X objective) that could be easily identified under microscope (Olympus IX-70) due to the cell characteristic, elongated shape (50-100 μm long). Myocyte viability was verified immediately before electrophysiological recordings by evaluating their reversible contractile responses to the application of noradrenaline from a pressure ejection pipette ([noradrenaline] in the pipette = 100 μM) [13]. The cell suspension was stored on ice, and the cells were used for patch-clamping up to 4 h after being isolated.

Electrophysiology

Oocytes were prepared for patch-clamp recordings as described [12]. Single-channel and macroscopic currents were recorded from inside-out (I/O) patches. For experiments with oocytes, both bath and electrode solutions contained (mM): 130 Kgluconate, 5 EGTA, 2.28 $MgCl_2$, 15 HEPES, pH 7.35. For experiments with myocytes, KCl substituted for Kgluconate. In all experiments, free Ca^{2+} in solution was adjusted to the desired value by adding $CaCl_2$. In the experiments where the free Ca^{2+}_i was set to ≥ 1 μM, 1.6 mM HEDTA was added. In all experiments, varying amounts of $CaCl_2$ were used to set the free Ca^{2+} at the desired level, keeping free Mg^{2+} constant at 1 mM [14]. Free Ca^{2+} and Mg^{2+} were calculated using Max Chelator (C. Patton, Stanford University, CA) and experimentally validated using Ca^{2+}-sensitive/reference electrodes (Corning) as described elsewhere [14].

Patch electrodes were pulled from glass capillaries (Drummond) as previously described [12]. The procedure gave tip resistances of 2-5 MΩ (for macropatch recordings) or 5-10 MΩ (for conventional I/O single channel recordings) when filled with electrode solution. An Ag/AgCl electrode was used as ground electrode. After excision from the oocyte, the inner side of the membrane patch was exposed to a stream of bath solution containing each agent at final concentration. Solutions were applied onto the patches using a DAD12 pressurized system (ALA Scientific) *via* a micropipette tip with an internal diameter of 100 μm. Experiments were carried out at room temperature (21°C).

Both macroscopic and unitary currents were acquired using an EPC8 (HEKA Electronics) amplifier, and digitized using a 1320 interface and pCLAMP8 or pCLAMP9 software (Molecular Devices). Macroscopic currents were evoked from I/O macropatches held at −80 mV by 200 ms-long, 10 mV depolarizing steps from −100 to +160 mV. Currents were low-pass filtered at 1 kHz with an 8-pole Bessel filter 902LPF (Frequency Devices) and sampled at 5 kHz. Average current amplitude was determined 175-200 ms after the start of the depolarizing step. Unitary currents were low-pass filtered at 7-10 kHz with an 8-pole Bessel filter and sampled at 35-50 kHz.

Macroscopic conductance (G)-V plots were fitted to a Boltzmann function of the type $G(V) =$ Gmax/1+exp[$(−V+V1/2)/k$]. Boltzmann fitting routines were run using the Levenberg-Marquardt algorithm to perform nonlinear least squares fits. Single-channel analysis was initially performed using pCLAMP9 (Molecular Devices). The product of the number of channels in the patch (N) and the probability that a channel is open (Po) was used as an index of the channel steady-state activity. NPo was calculated from the area under the curve of the Gaussian fit of all-points amplitude histograms [15-16]. NPo values were obtained from gap-free recording of single channel activity for 1 to 3 min under each condition.

Artery diameter determination

Middle cerebral arteries were isolated from adult male 8-12 week-old *KCNMB1* knockout and C57BL/6 (control) mice. Pressurization of arteries was performed as described [2]. Endothelium was removed by passing an air bubble into the vessel lumen for 90 sec. Diameter changes were monitored through an inverted microscope (Nikon Corp.), recorded on camera (Sanyo Electric Corp.), and stored on computer. Diameter data were acquired and analyzed using IonWizard 4.4 (IonOptics Corp.).

Pressurized arteries were extraluminally perfused with physiological saline solution (PSS) (composition in [2]) at a constant rate of 3.75 ml/min using a Dynamax RP-1 peristaltic pump (Rainin). Drugs were dissolved to make stock solutions (see Chemicals) and diluted in PSS to final concentration.

Chemicals

Except ethanol (American Bioanalytical) and iberiotoxin (Alomone), all chemicals were purchased from Sigma. Ethanol (100% pure) was freshly added to the bath solution immediately before experiments. Bath solution with urea iso-osmotically substituting for ethanol was used as control perfusion, with the solution osmolarity ranging 301-322 mM/kg (Wescor Vapro). Iberiotoxin was dissolved in distilled water to 20 μM and further diluted in PSS to final concentration.

Statistics

Electrophysiological and arterial diameter data were analyzed with pCLAMP 8.0 (Molecular Devices) and IonWizard 4.4 (IonOptics), respectively. Further analysis, plotting and fitting were conducted using Origin 7.0 (Originlab) and InStat 3.0 (GraphPad). Statistical analysis was conducted using one-way ANOVA and Bonferroni's multiple comparison test; significance was set at $P < 0.05$.

Results

Beta$_1$ subunits determine ethanol final action on recombinant cerebrovascular BK channels

Voltage-clamp electrophysiology on cell-free membrane patches after expression of recombinant BK channel subunits in *Xenopus* oocytes is a widely used approach to study ethanol actions on the BK channel complex [17]. Macroscopic BK currents mediated by cbv1 $\pm\beta_1$ subunits were evoked by depolarizing voltage steps (Materials and Methods) in I/O macropatches exposed to 0.3, 1, 3, 10, 30, and 100 μM Ca^{2+}_i, in absence and presence of 50 mM ethanol. This ethanol concentration corresponds to 3% (v/v) (or 30‰), which is found in circulation after heavy binge drinking [18] and reported to inhibit rat cerebrovascular myocyte BK currents [2]. Then, we obtained G/Gmax-V plots [15], from which we derived the voltage needed to achieve half-maximal conductance ($V_{1/2}$). At Ca^{2+}_i below 20 μM, ethanol caused BK current potentiation (Fig. 1A, top left two panels), which is evident by a reduction in $V_{1/2}$ (Fig. 1B). Our data also show that as Ca^{2+}_i increases, ethanol potentiation diminishes, turning to inhibition of current at $Ca^{2+}_i \geq 30$ μM (Fig. 1A, bottom left two panels). Thus, ethanol action on cerebrovascular myocyte homomeric cbv1-mediated current is a function of the channel-activating ligand (Ca^{2+}_i), resulting in alcohol final opposite effects: potentiation of current at resting, submicromolar Ca^{2+}_i *vs.* inhibition of current at high (>10 μM) Ca^{2+}_i.

The ethanol "opposing" effects on macroscopic current (Fig. 1A) were matched by the results describing drug behavior at single-channel resolution, with the alcohol increasing BK channel steady-state activity (NPo) at 0.3 μM Ca^{2+}_i while inhibiting activity at 30 μM Ca^{2+}_i (Suppl. Fig.1). These changes occurred without alteration of channel unitary current amplitude, indicating that ethanol action on cbv1 channels is limited to that of a gating modifier, with the alcohol final effect on current being determined by the Ca^{2+} level that is effectively sensed by the channel-forming subunit itself.

On the other hand, heteromeric cbv1+β_1–mediated currents were characterized by amplitudes significantly higher than those of homomeric cbv1, a reflection of the increase in the channel apparent Ca^{2+} sensitivity introduced by β_1 when co-expressed with slo1 [8,19-21]. Remarkably, β_1 subunits significantly shifted the "crossover" from ethanol-evoked activation to inhibition towards lower Ca^{2+}_i levels, namely, ≤ 3 μM Ca^{2+} (Fig. 1C). Therefore, at Ca^{2+} levels found in the vicinity of the BK channel during cerebral artery myocyte contraction (i.e., 4-30 μM) [22], the presence of β_1 subunits determines that ethanol inhibits BK current (Fig. 1D).

KCNMB1 ablation blunts ethanol-induced inhibition of native cerebrovascular BK channels and endothelium-independent vasoconstriction

To determine whether β_1 subunits determine ethanol final action on BK channels not only when the drug is probed on heterologously expressed recombinant channel subunits but also when the alcohol is evaluated on BK channels in their native environment, we next applied ethanol to native channels in myocytes freshly isolated from *wt* vs. *KCNMB1* knockout mice cerebral arteries. Recordings were obtained in I/O patches to match the configuration used with recombinants, with the membrane potential (−40 to −20mV) and Ca^{2+}_i (10 μM) set to levels reached near the native channel during myocyte contraction [22-23]. In myocytes from *wt* mouse, where native BK are heteromeric $\alpha+\beta_1$ complexes [9], 50 mM ethanol consistently caused a modest yet significant decrease in NPo (~ −26+/−9%; P<0.05), which was fully reversible (Fig. 2A,C). In contrast, ethanol caused a fully reversible increase in NPo when probed onto *KCNMB1* K/O myocytes (≈150% of control) (Figs. 2B,C). Ethanol responses from *wt* and *KCNMB1* K/O mouse match those obtained with recombinant cbv1+β_1 and cbv1, respectively (Suppl. Fig. 1), making it unlikely that putative factors compensatory to *KCNMB1* deletion could play a significant role in ethanol action on native BK current. More generally, the correspondence between oocyte and myocyte results seems to indicate that possible differences in proteolipid composition or membrane organization between frog oocyte and mouse myocyte membranes do not play a significant role in ethanol action on cerebral artery BK current. Collectively, data from recombinant and native BK channels indicate that BK accessory β_1 subunits are responsible for ethanol-induced current inhibition at physiological conditions of membrane voltage and Ca^{2+} found in the vicinity of the channel during cell contraction.

To determine the impact of β_1-mediated, ethanol inhibition of BK channels on organ function, we evaluated ethanol action on pressurized, de-endothelized, resistance-size cerebral arteries from *KCNMB1* knockout *vs. wt* mice. The presence/absence of a functional endothelium was determined from the vessel responses to endothelium-dependent and independent vasodilators, as described [2,24]. As control, we probed the vessels with 3 μM phenylephrine [25], which causes vasoconstriction without involving myocyte BK channels. Phenylephrine caused a similar reversible reduction in arterial diameter (~−10%) in *wt* and *KCNMB1* knockout animals (Fig. 3), indicating that *KCNMB1* ablation does not alter fundamental contractile and relaxation processes in the myocyte. In *wt* animals, 50 mM ethanol caused a robust decrease in diameter (average: −14±2%; Figs. 3A,C). Moreover, iberiotoxin (Ibtx) at concentrations that selectively block BK channels (100 nM) significantly prevented ethanol from causing endothelium-independent constriction (Suppl. Fig. 2). These data and those shown in Fig. 2 indicate that in the mouse species ethanol-induced cerebrovascular constriction is primarily determined by the alcohol inhibition of myocyte BK channels. This ethanol action is similar to that underlying rat cerebral artery constriction [2], buttressing the idea that the primary mechanisms mediating ethanol action on brain arteries are conserved among species.

In contrast to the *wt* mouse data, ethanol consistently (n=4) failed to constrict cerebral arteries from *KCNMB1* knockout mouse (Fig. 3B,C), occasionally evoking up to ~10% *increase* in diameter, which could be blocked by 100 nM Ibtx (not shown). These results indicate that smooth muscle BK β_1 subunits are the primary actuators of alcohol-induced, endothelium-independent cerebrovascular constriction, and buttress the idea that ethanol modulation of myocyte BK current determines ethanol final effect on cerebral artery diameter.

Discussion

Our study demonstrates that knocking out *KCNMB1* totally abolishes alcohol-induced constriction of pressurized, resistance-size cerebral arteries, these arteries remaining responsive to vasoconstrictors that do not act *via* BK inhibition. Consistent with the key role

of BK channels in controlling cerebrovascular tone [5] and buttressing their role as relevant targets of ethanol-induced cerebral artery constriction [2], *KCNMB1* deletion also suppresses acute ethanol inhibition of native BK channels in arterial myocytes exposed to levels of voltage and Ca^{2+} reached in the cerebral artery myocyte during contraction. Remarkably, in absence of *KCNMB1*, myocyte channels were not inhibited but activated by acute ethanol, a response that is characteristic of native BK channels in several neuronal preparations, whether the neuronally-abundant accessory β_4 subunit is present or not [26], vascular endothelium and other tissues [17,27-28] where *KCNMB1* expression is negligible [9].

Ethanol-induced cerebrovascular constriction *via* myocyte BK channels has been previously conceptualized into direct and indirect mechanisms. The former corresponds to a decrease in BK Po [2] resulting from alcohol actions on Ca^{2+}-driven gating of channel-forming slo1 [16]. The latter corresponds to ethanol inhibition of ryanodine receptor-mediated sparks [2], a vasodilatory local Ca^{2+} signal that activates BK channels in vascular smooth muscle [5]. In vascular myocytes, *KCNMB1* ablation has been reported to alter the coupling between Ca^{2+}-sparks and BK currents [29]. Thus, whether ethanol acts *via* direct or indirect mechanisms to cause cerebrovascular constriction, the central role of the BK β_1 subunit as a transducer that couples increases in $Ca^{2+}{}_i$ to BK activation explains the efficacy of *KCNMB1* deletion in totally blunting ethanol-induced inhibition of myocyte BK channels and the resulting cerebrovascular constriction. The molecular underpinnings of BK β_1 subunit-induced control of ethanol effect on BK channel gating remain to be addressed fully. However, data from recombinant cerebrovascular BK channels (Fig. 1) did demonstrate that the β_1-driven switch in the cbv1 channel's ethanol response from activation to inhibition corresponds to the β_1 subunit-induced leftward shift in the Ca^{2+} sensitivity of the channel complex. Using mouse brain recombinant slo1 (mslo; mbr5) channels, we recently studied the Ca^{2+}-dependence of ethanol effect on homomeric BK channels, and found that such dependence of alcohol action involves both the "Ca^{2+}-bowl" and the high-affinity Regulatory of Conductance for Potassium I (RCKI$_H$) domains for $Ca^{2+}{}_i$ recognition in mslo [16]. The similar Ca^{2+}-dependence of ethanol action on cbv1 (current study) and mslo currents [16] is consistent with the involvement of these slo1 Ca^{2+}-recognition sites in alcohol action, as the primary sequences of these two high affinity, $Ca^{2+}{}_i$-sensing sites show 100% identity between cbv1 (AAP82453) and mslo (mbr5; AAA39746).

Inhibition of mslo current is caused by ethanol favoring channel dwelling into a *Ca^{2+}-driven*, low-activity gating mode [16]. Notably, β_1 subunit's most notorious effect on BK current phenotype is a robust increase in the channel apparent Ca^{2+} sensitivity [19-20]. Conceivably, β_1 presence would favor ethanol's facilitation of channel dwelling into the low activity mode, which would be evident by a shift in the crossover from ethanol-induced activation to inhibition toward lower Ca^{2+} levels, as shown in Fig. 1. Consistent with this interpretation, 1) the neuronally-abundant β_4 subunit, which in contrast to β_1 does not modify the channel apparent Ca^{2+}-sensitivity at relevant levels of Ca^{2+} (~10 μM) [8], does not modify the crossover of ethanol actions on mslo(mbr5) channels [16]; and 2) β_1 subunits fail to modulate bovine aortic smooth muscle slo1 (bslo) NPo responses to ethanol when evaluated at 0.3 μM Ca^{2+} [14], a Ca^{2+} level at which β_1 modification of channel gating does not translate effectively in observable (N)Po modification [21].

Significance and health-relatedness

Present (Fig. 3) and previous data [2] show that ethanol-targeting of myocyte BK currents causes a 10-15% reduction in the diameter of resistance-size, cerebral arteries. This action would lead to ~30% reduction in cerebral blood flow, as diameter and blood flow are related by a third power relationship [30]. It is noteworthy that ethanol has been probed in middle cerebral arteries, which are particularly affected by stroke (www.strokecenter.org), using

concentrations obtained in circulation following heavy binge drinking. Binge drinking, the prevalent form of alcohol intake in developed countries, is an independent risk for stroke morbimortality [31]. Our current identification of the molecular target of ethanol-inhibition of myocyte BK current with resulting cerebrovascular constriction brings new insights into ethanol effect on BK channel, and opens a door for novel therapeutic approaches to treat cerebrovascular disease associated with alcohol intake.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

BK, large conductance, calcium- and voltage-gated potassium; N, number of channels present in the membrane patch; Po, channel open probability; STOC, Spontaneous Transient Outward Current; PSS, physiological saline solution; EtOH, ethanol.

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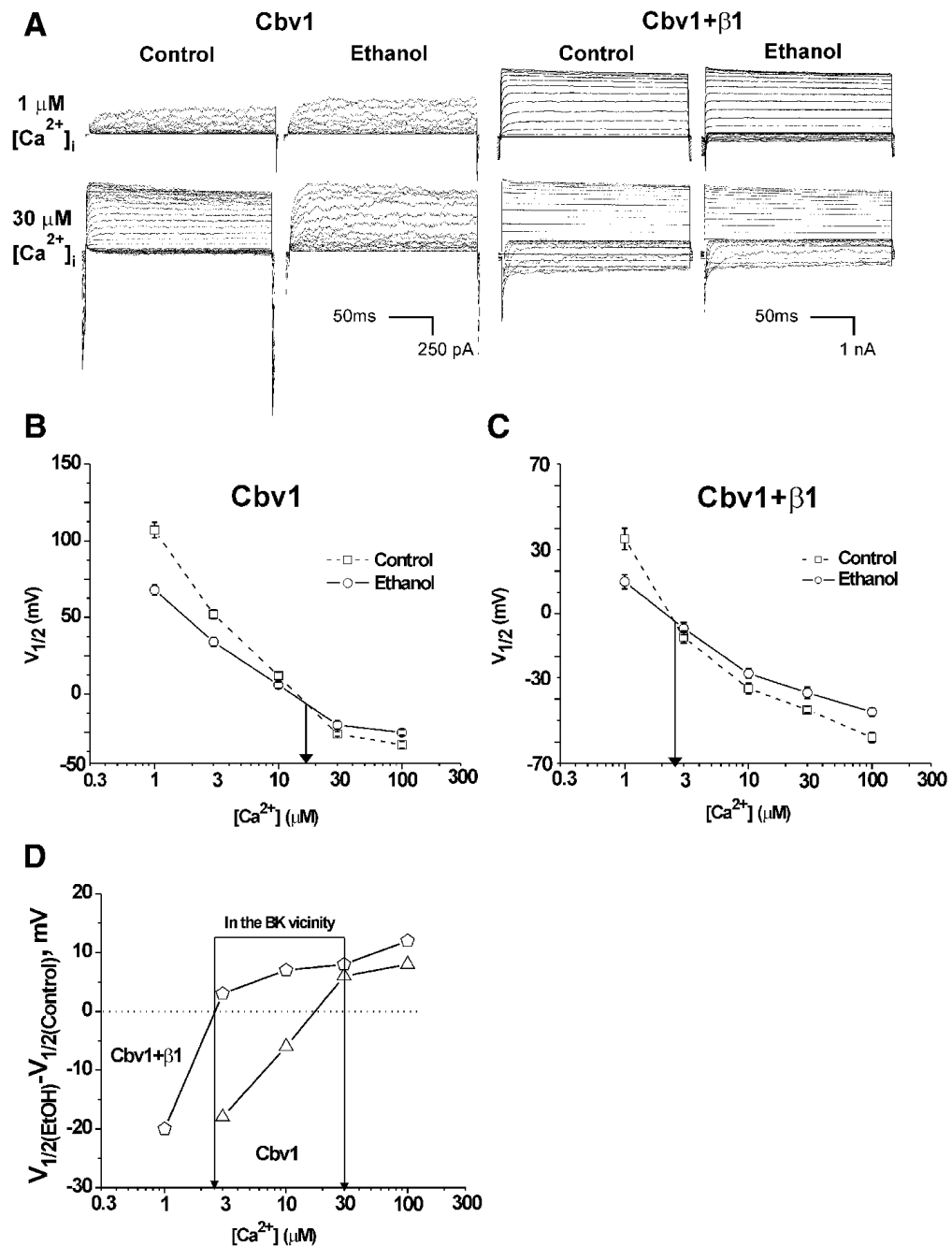
Fig. 1. β₁ subunits determine that ethanol inhibits recombinant BK current at physiological Ca²⁺ and voltage

A) BK currents from I/O macropatches recorded at 1 and 30 μM Ca²⁺ᵢ, in absence and presence of 50 mM ethanol, following cbv1±β₁ expression in *Xenopus* oocytes; **B)** β₁ shifts the activation-to-inhibition crossover of ethanol responses to <3 μM Ca²⁺ᵢ. Thus, at physiological Ca²⁺ᵢ found in the cerebral artery myocyte, ethanol causes inhibition of heteromeric cbv1 +β₁ current **(D)** (in D, points above and below the dotted line indicate inhibition and potentiation of current, respectively).
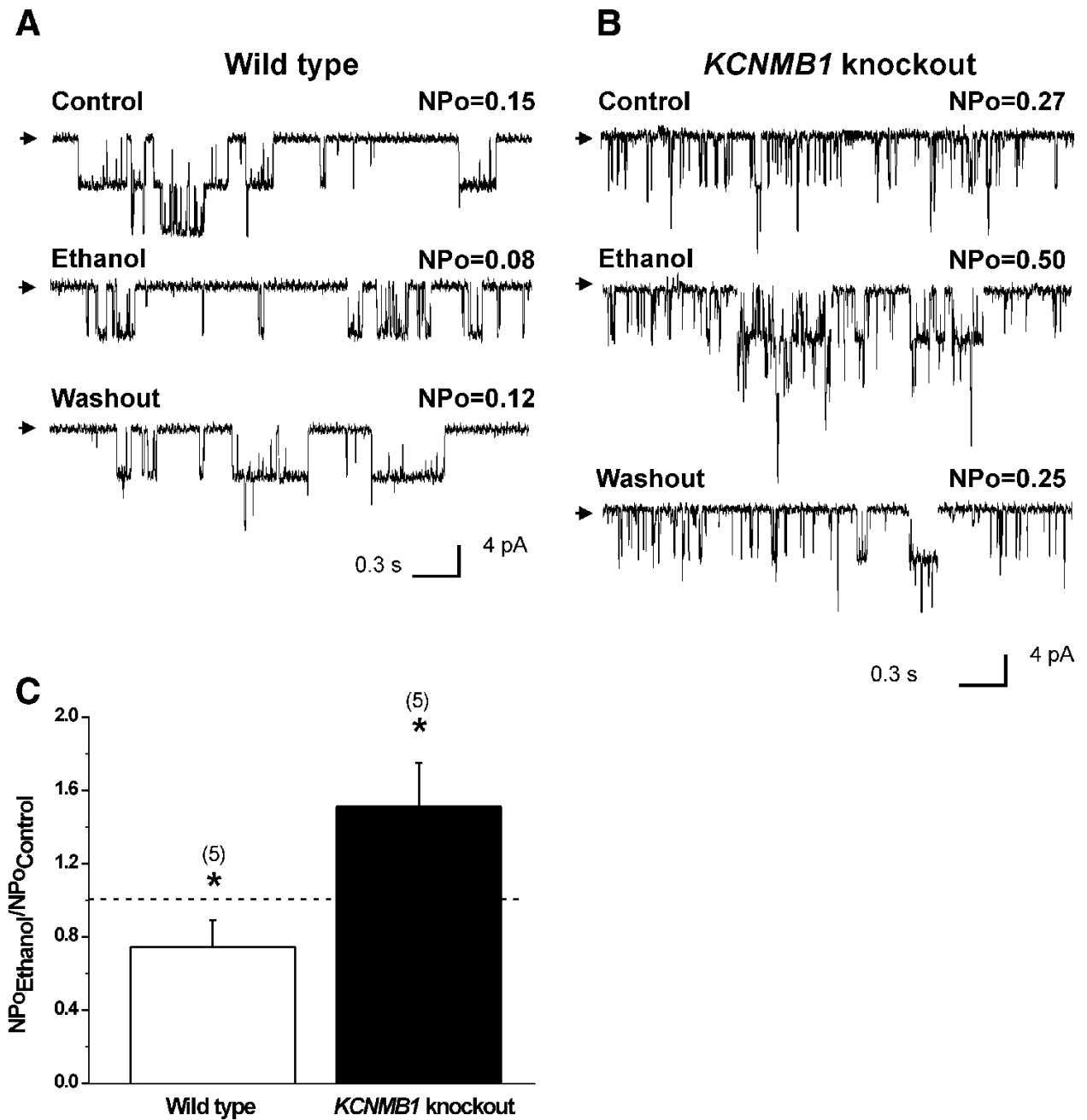
Fig. 2.
Single-channel recordings demonstrate that 50 mM ethanol inhibits cerebral artery myocyte BK channels in *wt* (**A**) while potentiating activity in *KCNMB1* knockout mice (**B**). Channel openings are downward deflections; arrows indicate the baseline. V=−30 mV; Ca^{2+}$_i$=10 μM; **C**) Averaged ethanol responses. **P<0.05, ethanol *vs.* control.
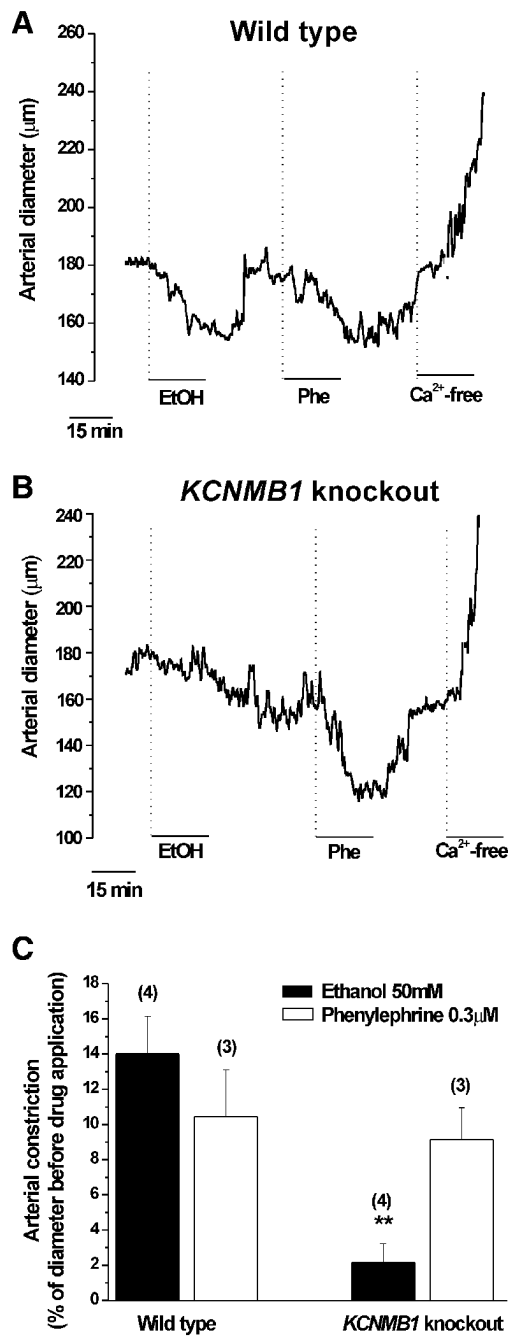
Fig. 3.
Diameter trace showing that ethanol constricts endothelium-denuded cerebral arteries in *wt* (**A**) but not in *KCNMB1* knockout mice (**B**). Phenylephrine (0.3 μM), a BK channel-independent vasoconstrictor, constricts cerebral arteries similarly in *wt* and *KCNMB1* knockout mice; **C)** Averaged vasoconstrictive responses. **P<0.01, *KCNMB1* knockout *vs. wt* mice.