Smooth Muscle Cholesterol Enables BK β1 Subunit-Mediated Channel Inhibition and Subsequent Vasoconstriction Evoked by Alcohol

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Objective—Hypercholesterolemia and alcohol drinking constitute independent risk factors for cerebrovascular disease. Alcohol constricts cerebral arteries in several species, including humans. This action results from inhibition of voltage- and calcium-gated potassium channels (BK) in vascular smooth muscle cells (VSMC). BK activity is also modulated by membrane cholesterol. We investigated whether VSMC cholesterol regulates ethanol actions on BK and cerebral arteries.

Methods and Results—After myogenic tone development, cholesterol depletion of rat, resistance-size cerebral arteries ablated ethanol-induced constriction, a result that was identical in intact and endothelium-free vessels. Cholesterol depletion reduced ethanol inhibition of BK whether the channel was studied in VSMC or after rat cerebral artery myocyte subunit (cbv1+β1) reconstitution into phospholipid bilayers. Homomeric cbv1 channels reconstituted into bilayers and VSMC BK from β1 knockout mice were both resistant to ethanol-induced inhibition. Moreover, arteries from β1 knockout mice failed to respond to ethanol even when VSMC cholesterol was kept unmodified. Remarkably, ethanol inhibition of cbv1+β1 in bilayers and wt mouse VSMC BK were drastically blunted by cholesterol depletion. Consistently, cholesterol depletion suppressed ethanol constriction of wt mouse arteries.

Conclusion—VSMC cholesterol and BK β1 are both required for ethanol inhibition of BK and the resulting cerebral artery constriction, with health-related implications for manipulating cholesterol levels in alcohol-induced cerebrovascular disease. (Arterioscler Thromb Vase Biol. 2011;31:00-00.)

Key Words: alcohol ■ cerebral circulation ■ ion channels ■ lipids ■ vascular muscle

Alcohol abuse is the third largest preventable cause of death, killing more than 100,000 Americans each year. Independently of any other factor, moderate-to-heavy episodic alcohol intake, such as during binge drinking, is associated with an increased risk for cerebrovascular spasm and death from stroke. Cerebrovascular disease associated with moderate-to-heavy alcohol intake is independent of beverage type and alcohol metabolism but is linked to pharmacological actions of ethanol (EtOH) itself. Moreover, acute EtOH administration at concentrations equivalent to blood alcohol levels (BAL) that represent legal intoxication (>17 mmol/L) constricts cerebral arteries in several species, including humans. However, the pathophysiology of EtOH-induced cerebrovascular disease in general, and cerebral artery constriction in particular, has remained largely unknown.

Using rat models, we previously demonstrated that EtOH-induced cerebral artery constriction resulted from the drug selective inhibition of large conductance, voltage- and Ca2+-gated K+ channels (BK) present in the plasmalemma of arterial myocytes. These BK consist of channel-forming α (slo1) and accessory (β1) subunits, the latter being particularly abundant in vascular and nonvascular smooth muscle but scarce in other tissues. Activation of cerebral artery myocyte BK generates outward K+ currents that lead to membrane hyperpolarization and myocyte relaxation and, thus, oppose vasoconstriction. EtOH-induced BK inhibition results in decreased artery diameter, an alcohol action that is independent of circulating and endothelial factors. Experimental conditions demonstrated that EtOH inhibition of cerebral artery BK is independent of alcohol metabolism by the cell and freely diffusible cytosolic signals. Rather, the rat cerebral artery myocyte (cbv1+β1) subunits and an isolated membrane environment suffice.

Cholesterol (CLR) is a major constituent of mammalian cell membranes. It determines overall membrane physical properties and affects the activity of transmembrane proteins, including BK. On one hand, BK subunits have been reported to localize within CLR-enriched membrane lipid “rafts.” CLR depletion increases BK current in coronary...
artery myocytes, suggesting that membrane CLR in vascular myocytes may regulate basal BK activity. On the other hand, modulation of membrane CLR content has been implicated in cell adaptation to chronic EtOH administration. Remarkably, the role of membrane CLR in acute EtOH actions and its impact on EtOH-induced alteration of vascular function remain unknown.

In the current study, we address the role of smooth muscle membrane CLR in EtOH action on cerebral artery myocyte BK function and resulting cerebral artery constriction. We used a variety of rat and mouse experimental models, including KCNMB1 knockout (KO) mice, to evaluate myogenic tone in both intact and endothelium-free arteries, as well as electrophysiological studies of cerebral artery myocyte BK both in native myocytes and following BK subunit reconstitution into artificial lipid bilayers. Our study demonstrates that membrane CLR and BK β1 are both absolutely required for EtOH blunting of channel function and drug-induced cerebral artery constriction.

Materials and Methods
Expanded materials and methods are available in the Supplemental Material, available online at http://atvb.ahajournals.org.

Cerebral Artery Diameter and Tone Determinations
Resistance-size, middle cerebral arteries were isolated from adult male Sprague-Dawley rats (~250 g) and 8- to 12-week-old KCNMB1 KO and C57BL/6 control mice as described elsewhere. Isolation of Arterial Myocytes From Rat and Mouse
Cells were freshly isolated as described. Modification of CLR Levels in Myocytes and Arteries
For CLR depletion, myocytes were incubated in 5 mmol/L methyl-β-cyclodextrin (MβCD)-containing bath solution for 20 minutes. For the same purpose, pressurized arteries were perfused for 60 minutes with physiological saline solution (PSS) containing 5 mmol/L MβCD. For CLR enrichment, bath solution and PSS contained 5 mmol/L MβCD + 0.625 mmol/L MCLR (8:1 molar ratio). To ensure MβCD saturation with CLR, the solution was vortexed and sonicated for 30 minutes at room temperature, then shaken at 37°C overnight. Times of myocyte incubation and artery perfusion with MβCD+CLR complex-containing solution were similar to those used with the CLR-depleting treatment (see above).

CLR and Protein Determinations
Arteries were de-endothelialized as previously described. Free CLR and total protein levels were determined using the Amplex Red Cholesterol Assay kit (Molecular Probes, Inc.) and the Pierce BCA protein assay kit (Thermo Scientific) following manufacturers’ instructions.

Electrophysiology Experiments on Native BK
Single-channel BK currents were recorded from excised, inside-out (I/O) membrane patches at V_m = −20 or −40 mV. Paxilline was applied to the extracellular side of the membrane patch in outside-out configuration. For experiments with rat and mouse myocytes, [Ca^{2+}]_l was set at 10 and 30 μmol/L, respectively.

Bilayer Experiments
BK reconstitution into and recording from artificial bilayers were performed as described.

Data Analysis
Statistical analysis was conducted using either 1-way ANOVA and Bonferroni’s multiple comparison test or paired Student t test, according to experimental design. Significance was set at P<0.05. Data were expressed as mean±SEM; (n)=number of arteries/myocytes/bilayers.

Results
CLR Levels Control Ethanol-Induced Constriction of Intact Cerebral Arteries
To evaluate whether CLR regulates EtOH-induced cerebral artery constriction, we isolated and pressurized rat middle cerebral arteries of 140- to 250-μm external diameter. These arteries control local vascular resistance and constitute a valid model for studying EtOH-induced cerebral artery constriction. At the end of the experiment each artery was perfused with Ca^{2+}-free PSS to determine passive diameter, which was used to calculate myogenic tone (Supplemental Figure 1A and Supplemental Material and Methods, available online at http://atvb.ahajournals.org). Perfusion of the artery with 60 mmol/L KCl reversibly decreased artery diameter by ~10% (Figure 1A–D). After complete washout of KCl, the artery was exposed to PSS containing 50 mmol/L EtOH, which corresponds to BALs found in circulation during moderate to heavy episodic alcohol intake and in the blood of alcoholics with stroke-like episodes. EtOH was applied for 13 minutes to evoke a steady arterial constriction, yet not long enough to damage arterial tissue. This duration of EtOH application also allowed us to compare current results with previous reports. In all cases, EtOH caused a significant decrease in artery diameter (up to 6%) when compared to pre-EtOH values (n=18; P<0.05) (Figure 1A–D). Constriction of cerebral arteries by direct and brief application of EtOH consistently reached 50% to 60% of the artery constriction by KCl (Figure 1E, column I). It is interesting to note that the response to KCl slowly declined over time (Figure 1A), evident from the reduced arterial response to a second KCl exposure applied 1 hour after the first (KCl II versus KCl I; Figure 1A–D). However, responses to EtOH remained steady whether the agent was applied for the first or second time (Figure 1A and 1D). Collectively, our data indicate that constriction of intact resistance-size cerebral arteries by EtOH occurs independently of circulating factors and alcohol metabolism by the body, with the cellular targets mediating such EtOH action not showing any evidence of EtOH-specific tolerance when challenged by the drug for a second time.

In a separate group of arteries, after assessing constriction of the vessel in response to KCl (KCl I and EtOH (EtOH I), we altered tissue CLR levels through pharmacological manipulations: To evoke CLR depletion, each artery was perfused with PSS containing 5 mmol/L MβCD for 1 hour. MβCD treatment routinely caused vasoconstriction, an action that fully vanished after MβCD washout (Figure 1B and Supplemental Figure 1B). This full and fast reversibility suggests that MβCD, aside from its CLR-depleting action, might directly modulate artery diameter. Investigation of possible mechanisms involved in this MβCD action are
Figure 1. Cholesterol (CLR) level-modifying treatments of intact cerebral arteries ablate ethanol (EtOH)-induced constriction. A, After myogenic tone development, either 60 mmol/L KCl or 50 mmol/L EtOH reversibly reduced diameter of arteries unexposed to CLR-modifying treatment (naïve CLR). Arterial responses to KCl and EtOH before (KCl I, EtOH I) and after (KCl II, EtOH II) CLR depletion (methyl-β-cyclodextrin; MβCD) (B) or enrichment (MβCD+CLR) (C). D, Averaged change in arterial diameter in response first (I) and second (II) KCl or EtOH applications. †Different from EtOH II tested on the artery with naïve CLR level (P<0.05). E, Averaged constriction by EtOH I and EtOH II as percentage of corresponding constriction by KCl. F, Averaged constriction by EtOH II as percentage of constriction by EtOH I. G, Superimposed arterial diameter responses to the second application of 1 μmol/L paxilline (paxilline II) to the CLR-naïve vs CLR-depleted vessel. H, Averaged change in arterial diameter in response to first (I) and second (II) applications of paxilline. *Different from arteries with naïve CLR (P<0.05); (n)=number of arteries. Vertical dashed lines indicate the start of drug application; hash shaded areas underscore changes in arterial diameter (area under the curve) evoked by drug application.
beyond the scope of this study. Because it remains unknown whether EtOH access and distribution into the membrane is affected by the dextrin, we washed out MβCD from the chamber with the artery before applying KCl and EtOH for a second time. After a 1-hour MβCD treatment, KCl reduced artery diameter by 7%, which is similar to the constriction evoked by a second KCl application in the MβCD-untreated vessels (KCl II in Figure 1D). Thus, in both MβCD-treated and -untreated arteries, the second KCl application identically reached ≈60% of the constriction evoked by a first KCl application to the naïve vessel. These results indicate that our MβCD treatment did not fully disrupt the contractile machinery of the cerebral artery.

In contrast, EtOH-induced constriction (Figure 1B; EtOH II) was drastically blunted by pre-exposure of the artery to MβCD: EtOH-induced decrease in diameter reached only about 15% of KCl-induced constriction (Figure 1D). The reduction in EtOH-induced constriction by MβCD treatment occurred well after myogenic tone recovered from direct exposure to MβCD (Figure 1B). Thus, such reduction is unlikely the result of direct antagonism of EtOH by MβCD. Instead, naïve CLR levels in the artery seemed to facilitate EtOH-induced constriction of intact, resistance-size cerebral arteries. Evaluation of passive artery diameter with Ca²⁺-free solution right after washout of EtOH showed that tone was well preserved in the artery that was previously subjected to MβCD treatment (Figure 1B versus 1A, and Supplemental Figure 1A). Data indicate that suppression of EtOH-induced cerebral artery constriction by pretreatment with MβCD was not a consequence of nonspecific loss of myogenic tone by the CLR-depleting treatment.

Finally, given that EtOH-induced cerebrovascular constriction is mediated by BK channels,⁶ we evaluated artery diameter responses to the selective BK channel blocker paxilline¹⁶ to test whether the loss of EtOH-induced vasocontraction after MβCD treatment was related to functional impairment of the BK channel population following exposure to MβCD. Application of 1 μmol/L paxilline (paxilline I) to the pressurized artery routinely caused a decrease (up to 8%) in arterial diameter (Figure 1H). This value is similar to results reported earlier on cerebrovasculature constriction evoked by selective block of BK channels.¹⁷ After 1 hour incubation of the artery in MβCD-free PSS, a second application of paxilline (paxilline II) resulted in ≈6% decrease in arterial diameter, suggesting no significant time-dependent change in BK channel responsiveness to the blocker. More important, arteries previously exposed to MβCD treatment responded to paxilline (paxilline II) with a constriction that was similar to those evoked in the same artery before CLR-depleting treatment (paxilline I), and to those from different, time-paired, arteries that were never exposed to MβCD (paxilline II) (Figure 1G and 1H). Thus, cerebral artery exposure to our MβCD-induced, CLR-depleting treatment appeared to impair selectively EtOH-induced arterial constriction.

We next determined whether CLR-enriching treatment of cerebral arteries amplified the EtOH-induced constriction observed in naïve arteries. Thus, another set of arteries was exposed to PSS containing MβCD+CLR.¹⁴ This MβCD+CLR treatment consistently dilated pressurized cerebral arteries (Figure 1C and Supplemental Figure 1B). The outcome was opposite to that evoked by MβCD alone and, thus, unlikely explained by a direct action of the dextrin on the artery. Rather, it should be attributed to CLR-delivery to the artery by the dextrin. Thus, our results support the novel concept that direct manipulation of CLR levels regulates diameter of cerebral arteries independently of CLR metabolism, circulating factors, and processes that are associated with long exposure to high CLR levels (eg, inflammation and plaque formation).

As found with MβCD treatment, however, KCl-induced constriction after CLR-enriching treatment resulted in a 6.5% decrease in artery diameter. This decrease did not significantly differ from the constriction in response to a second KCl application to the artery with naïve CLR levels (Figure 1D). On the other hand, 50 mmol/L EtOH applied to the CLR-enriched vessel (Figure 1C; EtOH II) resulted in only up to 3.5% reduction in artery diameter. Thus, the ratio of EtOH II/KCl II vasoconstrictions did not exceed 50%, which is not significantly different from the ratio of EtOH/KCl constrictrions evoked in the arteries with naïve CLR (Figure 1E). Collectively, results indicate that CLR-depleting treatment drastically blunted EtOH-induced constriction of intact, resistance-size cerebral arteries, whereas CLR-enriching treatment did not enhance the EtOH effect over that evoked in the naïve arteries (Figure 1F).

CLR Control of Cerebral Artery Responses to EtOH Is Independent of Endothelium

We next determined whether the regulation of cerebral artery responses to EtOH by CLR-manipulating treatments required a functional endothelium. Endothelium was removed prior to cannulation (Supplemental Materials and Methods) and its absence confirmed by the lack of responses in artery diameter to endothelium-dependent vasodilators, with arteries remaining responsive to endothelium-independent vasodilators.⁶,¹⁷

De-endothelialized arteries showed a myogenic tone that was not significantly different from intact vessels (Supplemental Figure IIA, available online at http://atvb.ahajournals.org). In addition, EtOH-induced constriction was identical (≈6% decrease in diameter) to that in arteries with intact endothelium (Figure 2D versus Figure 1D). This result underscores that EtOH-induced constriction of resistance-size cerebral arteries does not require a functional endothelium.

Several other outcomes in de-endothelialized vessels were undistinguishable from those in intact arteries: (1) EtOH constriction of endothelium-free vessels unexposed to CLR-manipulating treatment was very steady: 6% average reduction in diameter was observed with both first and second EtOH applications, the interval between applications being ≈1 hour (Figure 2A and 2D). This result suggests that, following an initial EtOH exposure, the smooth muscle targets that mediate EtOH-induced constriction do not suffer plastic changes within the hour; (2) exposure to 5 mmol/L MβCD rendered vasoconstriction, which disappeared on washout of the dextrin (Figure 2B; Supplemental Figure 1B); (3) the myogenic tone (Supplemental Figure IIA) was not significantly different from that in the artery unexposed to
Figure 2. Cholesterol (CLR) level-modifying treatments ablate ethanol (EtOH)-induced constriction independently of endothelium. 

A. After myogenic tone development, either 60 mmol/L KCl or 50 mmol/L EtOH reversibly reduced diameter of arteries unexposed to CLR-modifying treatment (naïve CLR). Arterial responses to KCl and EtOH before (KCl I, EtOH I) and after (KCl II, EtOH II) CLR depletion (methyl-β-cyclodextrin; MβCD) (B) or enrichment (MβCD + CLR) (C). 

D. Averaged change in arterial diameter in response first (I) and second (II) KCl and EtOH applications. †Different from EtOH II tested on the artery with naïve CLR level (P<0.05). 

E. Average constriction by EtOH II as a percentage of constriction by EtOH I. 

F. Superimposed arterial diameter responses to the second application of 1 μmol/L paxilline (paxilline II) to the CLR-naive vs CLR-depleted vessel. 

G. Averaged change in arterial diameter in response to first (I) and second (II) applications of paxilline. 

H. Free CLR content (in μg/mg of total tissue protein) after 1-hour incubation of de-endothelialized
MβCD. This result contrasts with recent findings showing that preincubation of de-endothelialized, rat cerebral arteries with 10 mmol/L MβCD for 2 hours prevents the artery from developing appropriate myogenic tone.18 Thus, the lower concentration and/or shorter exposition to MβCD applied in our study does not alter myogenic tone once developed; and finally, (4) MβCD treatment caused ablation of EtOH-induced constriction (EtOH II in Figure 2B, 2D, and 2E) without reducing the BK channel sensitivity to paxilline (paxilline II in Figure 2F and 2G). Therefore, modulation of responses to EtOH by CLR-depleting treatment does not result from nonspecific alteration of BK channel pharmacological responsiveness and occurs independently of endothelium.

On the other hand, preincubation of de-endothelialized arteries with MβCD + CLR complex rendered mild vasodilation (Supplemental Figure IIB), as well as a reduction in diameter (5%) in response to EtOH (Figure 2D). This effect was not statistically different from arteries untreated with MβCD or CLR (Figure 2D). Therefore, CLR-depleting treatment drastically blunted EtOH-induced constriction of de-endothelialized cerebral arteries, whereas CLR-enriching treatment did not result in further augmentation of EtOH-induced vasoconstriction from levels obtained in de-endothelialized arteries that had not been exposed to CLR-manipulating treatment.

To ensure that the observed results are not influenced by possible homeostatic compensations on CLR content in the artery that could occur on dextrin washout, we also tested the effect of EtOH on artery diameter when either MβCD or MβCD + CLR was still present in the PSS solution (Supplemental Figure IIIA–IIIC). The outcome of these experiments were identical to those in which EtOH II was applied after MβCD or MβCD + CLR was washed out (Supplemental Figure IIID and IIIE versus Figure 2D and 2E). Thus, modulation of alcohol constriction by CLR-depleting and CLR-enriching treatments remain effective after MβCD or MβCD + CLR is not present in the perfusion solution.

We next determined at which extent CLR-depleting and CLR-enriching treatments actually modified the CLR content of de-endothelialized, resistance-size cerebral arteries. We assessed the amount of free CLR per mg of total protein in de-endothelialized cerebral arteries by measuring free CLR and total protein levels using the Amplex Red Cholesterol and the Pierce BCA protein assays, respectively (Supplemental Material and Methods). Prior to these biochemical determinations, vessels were incubated for 1 hour in PSS alone, PSS with 5 mmol/L MβCD, or PSS with MβCD + CLR complexes. At the end of incubation, PSS-based solutions were removed and the necessary procedures were followed in accordance with the manufacturer’s kit instructions (please see Supplemental Material and Methods).

We found that naïve, free CLR levels in de-endothelialized arteries amounted to $\approx$10 μg/mg of total protein. Incubation of de-endothelialized cerebral arteries with 5 mmol/L MβCD significantly decreased free CLR levels to $\approx$ 5 μg/mg of protein (P<0.05), whereas treatment of arteries with MβCD-CLR complexes significantly increased free CLR levels above those in naïve vessels (P<0.05): $\approx$18 μg/mg of total protein (Figure 2H). These results are the first to demonstrate effective manipulation of CLR levels in de-endothelialized cerebral arteries by relatively brief incubations of the vessel with PSS containing MβCD or MβCD + CLR complexes. However, CLR depletion exerted an almost complete blunting of the endothelium-independent cerebrovascular constriction caused by EtOH, whereas CLR-enriching treatment exerted a very mild regulation of alcohol action (Figures 1F and 2E). This differential modulation of EtOH responses cannot be attributed to differential effectiveness of the 2 CLR-manipulating treatments in modifying the actual CLR content of the naïve vessel. Rather, these treatments differentially regulated the EtOH response of relevant targets in the de-endothelialized vessel. Considering that de-endothelialized arteries are largely composed of smooth muscle tissue,29 we thus hypothesize that EtOH-induced cerebral artery constriction requires a critical level of CLR in the smooth muscle itself.

**CLR-Ethanol Interaction Occurs at a Common Target: The Arterial Myocyte BK Complex**

We next focused on possible molecular targets and mechanisms in the vascular myocyte that could mediate the CLR–EtOH interaction. We concentrated on vascular myocyte BK channels because (1) they control cerebral artery myogenic tone,20 (2) they are considered primary mediator of EtOH-induced cerebrovascular constriction,4 and (3) their activity is modulated by CLR.9,10,21,22 We used patch-clamp recordings to evaluate CLR–EtOH actions on native BK in freshly isolated rat cerebral artery myocytes. Single-channel recordings were chosen over whole-cell currents to distinguish between possible modulation of unitary conductance versus channel steady-state activity (NPO), by exposure to EtOH and/or CLR [NPO = N(number of channels in the patch) × Po(open probability of 1 channel)]; for NPO determination see Supplemental Material and Methods. While maintaining physiological conditions of transmembrane voltage and Ca2+, the I/O patch configuration allowed us to evaluate EtOH/CLR actions on BK function in the absence of cell metabolism or channel modulators.

Considering the high effectiveness of CLR-depleting treatment to blunt smooth muscle-mediated cerebral artery constriction, cerebral artery myocytes were incubated in either control bath solution or bath solution containing 5 mmol/L MβCD before patch excision from the cell. Immediately after excision, each I/O patch was perfused with control bath solution 1 to 2 minutes to obtain basal BK function before EtOH application (Figure 3A,B, top traces). Preincubation of cerebral artery myocytes with MβCD significantly increased BK Po (Figure 3C) (for procedure on Po determination, see Supplemental Material and Methods). This fact suggests that...
Figure 3. Cholesterol (CLR) depletion abolishes ethanol (EtOH)-induced potassium channels (BK) inhibition. A, BK recordings and change in BK channel activity over time from an inside-out (I/O) patch excised from an arterial myocyte with naïve cholesterol (CLR content) or (B) CLR-depleted. Channel activity is shown before (control), during, and after (washout) exposure to bath solution containing 50 mmol/L EtOH (solution composition in Materials and Methods). Channel openings indicate downward deflections; arrows, baseline (all channels are closed); dotted lines, channel open level. V_m = -20 mV, free Ca^{2+} = 10 μmol/L. C, BK open probability (Po) from I/O patches from myocytes with naïve vs depleted CLR. Po was obtained after determination of N and calculation of NPo [see Supplemental Material and Methods, available online at http://atvb.ahajournals.org]. D, BK current from arterial myocyte after CLR depletion exhibits paxilline sensitivity. V_m = -40 mV, free Ca^{2+} = 10 μmol/L. E, Average changes in BK activity (NPo). A dotted line underscores control (predrug) NPo. *Different from control (P<0.05); (n) = number of membrane patches tested.
myocyte membrane CLR exerts a tonic inhibitory influence on BK activity.

Because in the experiments on cerebral artery tone (Figures 1 and 2) maximal EtOH-induced constriction was observed ~7 to 10 minutes of EtOH perfusion, we exposed the membrane patch to EtOH-containing versus control solution and recorded BK currents for 10 minutes. NPo versus time plots in Figure 3A and B show that EtOH application causes a transient activation of BK channels (in ~1.5 minutes), as reported with native channels in cerebral artery myocytes and recombinant hSLO1 in artificial lipid bilayers.6,21 However, as reported with native cerebrovascular channels,6 transient activation is followed by a robust and sustained decrease in channel activity that only recedes on EtOH withdrawal. Because a decrease in cerebrovascular BK NPo effectively determines EtOH-induced cerebral artery constriction,6,8 we measured EtOH action at a time point where it reached steady inhibition of channel function, ie, 7 to 10 minutes after initiation of perfusion with EtOH-containing solution. In myocytes not subjected to CLR-depleting treatment, 50 mmol/L EtOH consistently caused a significant reduction in BK NPo, which reached 65% of pre-EtOH levels. This EtOH action was fully reversible on washing the membrane patch with EtOH-free solution (Figure 3A and 3E). EtOH-induced reduction in BK NPo was not accompanied by any change in unitary current amplitude (Figure 3A, top versus middle traces), confirming a previous report.6 The most striking finding was that EtOH inhibition of cerebral artery BK was totally blunted in myocytes previously exposed to CLR-depleting treatment (Figure 3B and 3E). This difference in EtOH action on naïve versus CLR-depleted myocytes cannot be attributed to changes in NPo that occurred with time after patch-excision: In both untreated and CLR-depleted myocytes, BK NPo in the I/O patch did not change ~10% from its initial level over 11 minutes of continuous recording in EtOH-free solution (Figure 3A and 3B). Also, CLR depletion treatment did not affect ability of selective BK channel blocker paxilline to reduce NPo (Figure 3D and 3E). While CLR depletion increased BK activity, such treatment blunted reduction of NPo by EtOH. Thus, it seems that membrane CLR facilitates EtOH inhibition of cerebral myocyte BK. It should be underscored that CLR–EtOH synergism is found in cell-free myocyte membranes, which indicates that CLR regulation of EtOH action is independent of freely diffusible cytosolic signals and cell metabolism of CLR or EtOH.

**BK β1 Subunit Augments CLR-Ethanol Synergistic Inhibition of Cerebral Artery Smooth Muscle BK**

Cerebral artery myocyte BK consists of channel-forming slo1 (eg, cbv1) and regulatory β1 subunits.7 The latter confers a distinct ionic current and pharmacology phenotype, including sensitivity to choline steroids.21 To determine which BK protein(s) is involved in the CLR–EtOH interaction, we determined the EtOH responses of homomeric cbv1 versus heteromeric cbv1+β1 complexes following channel reconstitution into 3:1 POPE:POPS (w/w) bilayers in the absence and presence of 23 mol% CLR. This binary phospholipid bilayer supports both CLR10,21,24 and EtOH modulation21 of reconstituted BK. The CLR molar fraction chosen was close to that found in native plasma membranes of mammalian cells25 and corresponded to the EC50 for CLR-induced inhibition of BK in this bilayer type.24 Unitary current events from cbv1 and cbv1+β1 channels displayed distinct features of BK openings: large ion current amplitude (14 pA at 0 mV in 300/300 mmol/L [K+]o/[K+]i); and increased activity as the voltage was made more positive and/or when Ca2+, was increased, as previously demonstrated in the same bilayer type.24 For data acquisition and further analysis we used bilayers that contained 1 channel. Thus, single-channel Po was used as an index of BK activity. On channel protein incorporation into bilayer, membrane voltage was briefly held at −60 mV to confirm subunit composition within the channel complex. At this voltage and [Ca2+], (10 μmol/L), cbv1 had Po and open times noticeably shorter than those from cbv1+β1, underscoring that the homomeric versus heteromeric nature of recombinant BK from cerebral artery myocytes can be easily distinguished based on unitary current phenotype.24

Application of 50 mmol/L EtOH to the cis chamber (cytosolic side of the channel) of the bilayer containing 23 mol% CLR consistently caused a robust decrease in cbv1+β1 Po, which reached 65% of control, pre-EtOH values (P<0.01) (Figure 4A,C). This EtOH-induced decrease in channel activity was quantitatively similar to that observed with native BK (Figure 3A and 3E), which are thought to consist of α+β1 heteromers.7 Results from native channels and cbv1+β1 recombinant proteins reconstituted into artificial CLR-containing bilayers indicate that EtOH inhibition of cerebrovascular BK is sustained in a bare proteolipid environment. The quantitative correspondence in EtOH-induced reduction in NPo (myocyte native channel) and Po (cbv1+β1 in bilayers) suggest that EtOH action results solely from a reduction in Po. Finally, it should be noted that EtOH inhibition of cbv1+β1 channels in the presence of CLR cannot be attributed to the reduction of Po caused by CLR itself; CLR alone reduced Po by ~10%, whereas EtOH in the presence of CLR rendered a reduction in Po of ~35% (P<0.05) (Figure 4C).

On the other hand, EtOH routinely failed to reduce cbv1+β1 Po in CLR-free bilayers (Figure 4B and 4C). Channels in the CLR-free bilayer, however, retained their sensitivity to 1 μmol/L paxilline (Supplemental Figure IV, available online at http://atvb.ahajournals.org), indicating that CLR is not necessary for paxilline-induced reduction of BK channel activity. Thus, our results seem to indicate that bilayer CLR rather selectively facilitates EtOH inhibition of cerebral artery BK channels. This inhibition does not require the complex proteolipid environment and signaling present in native myocytes. Rather, such modulation may occur at the BK proteins themselves.

Extending previous data,8 current results demonstrated that the cerebrovascular BK β1 protein drastically facilitated EtOH inhibition of BK (Figure 4A versus 4D; also: Figure 4C versus 4F). However, EtOH failed to inhibit cbv1+β1 in the CLR-free bilayer (Figure 4B and 4C), a result identical to that from cbv1 (Figure 4E and 4F). Therefore, the presence of β1...
Figure 4. Cholesterol (CLR) is required for potassium channels (BK) β1-mediated inhibition of cerebral artery BK reconstituted into phospholipid bilayers. A, Following channel reconstitution into 23 mol% CLR-containing POPE:POPS (3:1 w/w) bilayer, 50 mmol/L ethanol (EtOH) decreased cbv1 + β1 open probability (Po) from pre-EtOH values. B, In CLR-free POPE:POPS (3:1 w/w) bilayer, 50 mmol/L ethanol (EtOH) failed to modify cbv1 + β1 Po from pre-EtOH values. C, Average data underscore that cbv1 inhibition by EtOH requires bilayer CLR. *Different from Po in CLR-free bilayer in absence of EtOH (P < 0.05). In CLR-containing (D) and CLR-free (E) bilayers, cbv1 Po remained the same before and after EtOH. F, Average data from cbv1. In A, B, D, and E, channel openings indicate upward deflections; arrows, baseline. In D and F, n = 4 bilayers. Vm = 0 mV; free Ca2+ = 10 μmol/L.
was not sufficient to ensure EtOH inhibition of BK, but membrane CLR was required.

In contrast to EtOH inhibition of cerebrovascular BK, CLR inhibition of channel activity seemed to be unaffected by coexpression of β1 cloned from cerebral artery myocytes (Figure 4B.A versus 4E.D, 4C versus D), a result that confirmed a previous finding from cbv1 coexpressed with bovine tracheal smooth muscle β1.24 In summary, data from binary phospholipid bilayer indicate that, while CLR inhibition of cerebrovascular BK is β1-independent, CLR and BK β1 are both required for EtOH to inhibit BK.

We next determined whether the critical role of membrane CLR-BK β1 in EtOH action was preserved when the channel was studied in its native membrane environment. Thus, I/O recordings under physiological conditions of voltage and Ca2+ were conducted on myocytes freshly isolated from wt C57BL/6 versus KCNMB1 KO mice. Before EtOH application, a separate group of myocytes was exposed to the CLR-depleting treatment described above for rat myocytes. Depletion of membrane CLR resulted in basal BK Po values that were slightly, but consistently, higher than those from myocyte membranes unexposed to CLR-depleting treatment (Figure 5B versus 5A; Supplemental Figure V, online at http://atvb.ahajournals.org). This result is in agreement with data from rat myocyte BK (Figure 3C), and cbv1+β1 reconstituted into artificial bilayers where the presence of CLR the bilayer reduced cbv1+β1 Po (Figure 4A–4C). Findings from rat and mouse myocytes and binary bilayers put forward the idea that targets common to all these systems should mediate CLR action.

We failed to detect any significant difference in BK Po increase by CLR-depleting treatment when we compared wt versus KCNMB1 KO myocytes: In both cases, NPo increased ≈12% (Figure 5A and 5B versus 5D and 5E). This result indicated that whether the cerebrovascular BK was present in its native myocyte membrane or reconstituted into a bare lipid environment, channel-forming (eg, cbv1) subunits were sufficient to provide CLR sensitivity. Moreover, any possible mechanism compensatory to KCNMB1 ablation did not drastically alter the CLR sensitivity of the channel.

As demonstrated with BK from rat cerebral artery myocytes (Figure 3E), BK NPo from wt mice was drastically and reversibly reduced by 50 mmol/L EtOH (Figure 5A and 5C). In contrast, EtOH inhibition of BK activity was consistently reversibly reduced by 50 mmol/L EtOH (Figure 5A and 5C). Thus, β1, whether in native vascular myocyte membrane or inserted into an artificial lipid bilayer, critically facilitates EtOH inhibition of BK.

Ethanol-induced channel inhibition in wt mouse myocytes totally vanished after exposing the myocyte membrane to CLR-depleting treatment (Figure 5B versus 5A and 5C), whereas an EtOH negligible effect was found in both naïve and CLR-depleted myocytes from KCNMB1 KO mice (Figure 5D–5F). Therefore, cerebral artery myocyte membrane CLR was required for BK β1 subunit-mediated inhibition of BK by EtOH. Finally, it is noteworthy that our EtOH results on BK from wt (β1-containing) mouse cerebral artery myocytes were practically identical to those from rat cerebral artery myocytes, indicating that the critical role of CLR in EtOH-BK β1 interaction was common to more than 1 species.

CLR Regulation of the BK β1 Subunit-EtOH Interaction Has a Profound Impact on Organ Function

To test the role of BK β1 in CLR–EtOH interaction at the organ level, we used de-endothelialized, pressurized cerebral arteries from wt C57BL6 versus KCNMB1 KO mice. We probed artery diameter with 50 mmol/L EtOH when CLR level in the arterial wall was kept unmodified (naïve) or after CLR-depleting treatment (20 minutes perfusion of 5 mmol/L MβCD in PSS). In all cases, myogenic tone was determined as previously described for rat cerebral arteries. As found with rat arteries (Figures 1 and 2), EtOH caused a significant reduction in wt mouse artery diameter (up to 14%) from pre-EtOH levels (Figure 6A, C). As also found in rats, 5 mmol/L MβCD consistently decreased cerebral artery diameter in wt mice (Supplemental Figure VIB, available online at http://atvb.ahajournals.org). Ethanol-induced constriction, however, was lost after wt mouse arteries were pre-exposed to CLR-depleting treatment (Figure 6B and 6C), underscoring that CLR levels found in natural membranes are required for this EtOH action. Our study indicates that endothelium-independent, EtOH constriction of resistance-size cerebral arteries was conserved between rat and mouse, in both species being enabled by the levels of CLR naturally present in the myocyte membrane.

In contrast to data from wt mice, de-endothelialized arteries from KCNMB1 KO mice did not constrict in response to 50 mmol/L EtOH, whether or not they had been exposed previously to CLR-depleting treatment (Figure 6D–6F). However, arteries from wt and KCNMB1 KO mice similarly constricted in response to MβCD incubation (Supplemental Figure VIB). Moreover, we did not detect any significant difference in the myogenic tone of arteries from both groups of mice (Supplemental Figure VIA). Therefore, the drastic difference in the ability of EtOH to constrict wt versus KCNMB1 KO arteries did not result from a nonselective disruption of arterial contractility or alterations in MβCD sensitivity in the KCNMB1 KO model. Instead, lack of EtOH sensitivity in KCNMB1 KO arteries should be attributed to the specific absence of BK β1. The lack of EtOH action on wt (β1-containing) arteries when CLR is depleted highlights the fundamental role of smooth muscle membrane CLR in BK β1 subunit-mediated EtOH vasoconstriction.

Discussion

We provide the first evidence that arterial smooth muscle membrane CLR critically controls alcohol responses of resistance-size, middle cerebral arteries in 2 rodent models widely used for studying EtOH-induced cerebral artery constriction in humans.4,6,8 Independent of circulating or endothelial factors, artery exposure to EtOH for several minutes leads to an average decrease in diameter of at least 5.5% (in rats) and up to 14% (in mice). Both numbers are consistent with previous reports on rat and mouse models, respec-
Figure 5. Cholesterol (CLR) is required for potassium channels (BK) β1 subunit-mediated inhibition of BK in cerebral artery myocytes. Unitary current recordings in inside-out (I/O) patches from wt C57BL/6 mouse arterial myocyte having naïve (A) and CLR-depleted levels (B). Channel activity (NPo) is depicted before (top), during (middle), and after (bottom) bath application of 50 mmol/L ethanol (EtOH). C, Average results from wt C57BL/6 mice. *Different from pre-EtOH NPo (P<0.05). Unitary current recordings in I/O patches from KCNMB1 knockout (KO) mouse arterial myocytes having naïve CLR (D) or CLR-depleted levels (E) before (top), during (middle), and after (bottom) bath application of 50 mmol/L EtOH. F, Average results from KCNMB1 KO mice. In A, B, and D, openings indicate downward deflections; arrows, baseline; dotted lines, open channel levels. In C and E, a dotted line underscores NPo before EtOH application. In all cases: $V_m=-20$ mV; free Ca$^{2+}=30$ μmol/L; (n)=number of membrane patches tested.
Figure 6. Cholesterol (CLR) depletion eliminates ethanol (EtOH)-induced vasoconstriction in potassium channels (BK) β1-containing mouse cerebral arteries. Diameter traces of pressurized, de-endothelized arteries from wt C57BL/6 mice show that 50 mmol/L EtOH constricted arteries with naïve CLR content (A) but fails to do so in CLR-depleted (methyl-β-cyclodextrin; MβCD) arteries (B). C, Average data from wt C57BL/6 mice. **Different from arteries with naïve CLR (P<0.01). Data from KCNMB1 knockout (KO) mice show that 50 mmol/L EtOH failed to evoke constriction of CLR-naive (D) or CLR-depleted (E) arteries. D, Average data from KCNMB1 KO mice. In C and F, (n)=number of arteries. Vertical dashed lines indicate the start of drug application; hash shaded areas underscore changes in arterial diameter (area under the curve) evoked by drug application.
According to Poiseille’s law, liquid flow in a tube is proportional to radius by a 4th power. Moreover, experimental studies on cerebral vessels demonstrated that artery diameter was related to blood flow by a factor of 3 to 4.26 Therefore, EtOH action on cerebral artery diameter would lead to at least a 17% decrease in cerebral regional blood flow. The EtOH concentration chosen for our experiments matched BAL found in circulation during moderate-heavy episodic alcohol intake, such as in binge drinking, and found in the blood of alcoholics with stroke-like episodes.1,3 The experimental conditions under which we addressed modulation of EtOH action by CLR manipulation precluded involvement of circulating factors and processes that require long-term exposure of the vessel to CLR modulation. Moreover, we found that the loss of EtOH-induced cerebral artery constriction by CLR depletion was identical in intact and de-endothelized vessels (Figures 1 and 2). Thus, this CLR–alcohol interaction impacting organ function occurs at cell targets that very likely reside in the cerebral artery smooth muscle itself. Whether using intact or de-endothelized arteries, CLR depletion did not drastically affect the vessel response to KCl-induced vasoconstriction or Ca2+–free solution-induced vasodilation (Figures 1 and 2). These results underscore that CLR depletion does not disrupt the smooth muscle contractile machinery but selectively impairs EtOH action on specific targets in the cerebral artery myocyte. Indeed, our data from KCNB1 KO versus C57BL/6 (control) mice document for the first time that CLR modulation of endothelium-independent constriction of cerebral arteries in response to EtOH has an absolute requirement for the BK accessory β1 subunit (Figure 6). It is noteworthy that this subunit is particularly abundant in vascular smooth muscle where it resides tightly associated to the BK pore-forming slo1 subunit.7 Thus, the CLR–EtOH interaction on BK channels should be particularly relevant to vascular pathophysiology (see below).

Two complementary approaches were used to document the role of membrane CLR in controlling EtOH responses of β1 subunit-containing BK: (1) exposure of native myocytes (rat and mouse) to a CLR-depleting treatment that proved effective to drastically decrease the actual content of CLR in de-endothelized cerebral arteries (Figure 2H) and blunt rat (Figures 1 and 2) and wt mouse (Figure 6) vasoconstriction in response to EtOH; and (2) evaluation of EtOH action on recombinant channels reconstituted into artificial binary bilayers in the absence and presence of CLR at a molar fraction (23 mol%) close to CLR levels found in the plasma membrane of mammals.25 Our results from native channels and recombinant cbv1+β1 reconstituted into artificial CLR-containing bilayers indicate that EtOH inhibition of cerebrovascular BK requires the presence of CLR in the membrane. CLR facilitation of this EtOH inhibitory action, however, does not result from the summation of the individual action of each agent on basal channel activity (Figure 4C). CLR–EtOH synergism cannot be explained by CLR facilitation of EtOH partitioning in the membrane either: changes in lipid phase behavior and nuclear magnetic resonance data collectively indicate that EtOH partition into a phospholipid bilayer is actually decreased by CLR presence in the system, as fully discussed elsewhere.21 Thus, CLR–EtOH synergism to reduce BK Po must result from pharmacodynamic interactions between the 2 modulators. Moreover, this synergism likely results from allosteric interaction via differential contribution of the BK protein subunits to each modulator action (see Supplemental Discussion, available online at http://atvb.ahajournals.org).

Interestingly, the ability of CLR to modulate ion channel basal function and pharmacology differentially seems to be a widespread phenomenon. For instance, CLR depletion of mouse skeletal fibers inhibits the activity of L-type Ca2+-channels but facilitates their activation by Bay-K8644.27 Increases in CLR levels do not change conduction properties of voltage-gated Na+ channels but significantly inhibit phenobarbital-induced block of this channel.28 Finally, increase in membrane CLR increases the efficacy of nonsteroidal activators of GABA-A receptors, such as propofol, flunitrazepam, and pentobarbitone, while decreasing the action of steroid activators, such as pregnanolone and alphalone.29 In most of these studies, however, the mechanisms and channel subunits underlying CLR-drug interaction on ion channel function have remained largely unknown. As found for CLR–EtOH modulation of cerebral artery tone, our results from excised-patch membranes and artificial bilayers document that CLR–EtOH interaction on BK activity is independent of circulating and endothelial factors, cell metabolism of CLR or EtOH, intracellular organelles, and freely diffusible cytosolic signaling. Moreover, the identical results obtained with EtOH on native myocytes from mouse and rat exposed to CLR-depleting treatment on one hand and CLR-free bilayers on the other indicate that CLR modulation of EtOH action does not require the complex lipid and protein cytoarchitecture of native membranes. Instead, a system with a minimum of targets represented by 2-species phospholipids and cbv1+β1 subunits suffice. While we previously demonstrated an important role for BK β1 in determining BK channel responses to EtOH,4 the current set of data establish that the presence of BK β1 is not sufficient to ensure EtOH inhibition of BK and consequent cerebral artery constriction but that membrane CLR is required.

A direct modulation of BK β1-mediated vasoconstriction in response to EtOH by smooth muscle membrane CLR may have wide implications for human vascular pathophysiology and disease. First, changes in membrane CLR levels in different tissues and cell types correlate with variations in CLR plasma levels.30 Second, elevated CLR plasma levels31 and moderate-to-heavy EtOH drinking32 are both associated with vascular smooth muscle pathology in humans. On one hand, we demonstrated in this study that decreasing CLR levels in cerebral artery myocyte membranes blunted BK-mediated alcohol-induced cerebrovascular constriction, the latter contributing to cerebrovascular pathology associated with alcohol intake.3 Because CLR-lowering therapy by statins is known to effectively reduce CLR levels in normocholesterolemic subjects,33 it is possible to speculate that statins might be of use in mitigating alcohol-induced cerebral artery constriction and its consequences in normocholesterolemic patients. On the other hand, we demonstrated that an increase in cerebral artery myocyte membrane CLR did not further augment EtOH-induced vasoconstriction. Thus, pos-
sible vascular benefits of CLR-lowering intervention in hypercholesterolemic patients who are binge drinkers would result from a variety of CLR-driven pathophysiological mechanisms other than targeting myocyte BK channels.

Finally, our study clearly demonstrated that myocyte membrane CLR is not sufficient to support EtOH inhibition of cerebral artery myocytes but BK β1 subunits are needed for drug action. Interestingly, by using an animal model (e.g., rat coronary artery), it has been proven that ageing causes a functional decline of BK β1 subunits.34 Considering the key role of this subunit in EtOH-induced arterial constriction6 (see also current data), optimal levels of BK β1 in young people would make this population particularly susceptible to EtOH-induced vasoconstriction. As mentioned above, our study was conducted using EtOH levels that match BAL obtained in humans after binge drinking, which is the prevalent form of alcohol intake in youth, college students in particular.35 Our data might also lead to the idea that ablation of BK β1 subunits would be beneficial in alcohol-driven vascular pathology. However, these possible benefits could be counteracted by the cardiovascular and renal alterations likely to result from lack of BK β1 function, as evident from the KCNMB1 KO animal model.36,37

In conclusion, we have demonstrated for the first time that, independent of circulating, endothelial, and inflammatory mediators, a decrease in smooth muscle membrane CLR blunts BK β1 subunit-mediated cerebrovascular constriction in response to EtOH. BK β1 and CLR are both required for EtOH effect, their influence is extending from highly simplified systems such as an artificial binary bilayer to organ function, i.e., the pressurized, endothelium-intact cerebral artery. Mechanistically, however, the influence of CLR and EtOH on basal channel activity is differentially controlled by the BK subunits via Ca2+-independent and Ca2+-dependent gating, respectively.

Acknowledgments
The authors thank Dr David Armbruster for critical reading of the manuscript.

Sources of Funding
This work was supported by an Alcoholic Beverage Medical Research Foundation grant for New Investigator, NIH Support Opportunity for Addiction Research for New Investigators R03 AA020184 (A.B.), and NIH Merit Award R37 AA11560 (A.D.).

Disclosures
None.

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Smooth Muscle Cholesterol Enables BK β1 Subunit-Mediated Channel Inhibition and Subsequent Vasoconstriction Evoked by Alcohol
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Arterioscler Thromb Vasc Biol. published online August 25, 2011;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2011/08/25/ATVBAHA.111.233965

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Supplement Material

Materials and Methods

Cerebral artery diameter and tone determinations. Adult male Sprague-Dawley rats (≈250 g) and 8- to 12-week-old KCNMB1 KO and C57BL/6 control mice were decapitated using a guillotine and sharp scissors, respectively. These procedures were approved by the Institutional Animal Care and Use Committee from The University of Tennessee Health Science Center, an AAALAC-accredited institution. Middle cerebral arteries were isolated on ice under microscope (Nikon SMZ645) from rat or mouse brain and cut into 1 to 2 mm-long segments. A segment was cannulated at each end in a temperature-controlled, custom-made perfusion chamber. Using a Dynamax RP-1 peristaltic pump (Rainin Instr.), the chamber was continuously perfused at a rate of 3.75 ml/min with PSS (mM): 119 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 1.6 CaCl₂, 1.2 MgSO₄, 0.023 EDTA, 11 glucose, 24 NaHCO₃. PSS was equilibrated at pH 7.4 with a 21/5/74% mix of O₂/CO₂/N₂ and maintained at 35-37°C. Arteries were monitored with a CCD camera (Sanyo VCB-3512T) attached to an inverted microscope (Nikon Eclipse TS100). The artery external wall diameter was measured using the automatic edge-detection function of IonWizard software (IonOptics) and digitized at 1 Hz using a personal computer. Steady-state changes in intravascular pressure were achieved by elevating an attached reservoir filled with PSS and were monitored using a pressure transducer (Living Systems Instr.). Arteries were first incubated at an intravascular pressure of 10 mm Hg for 10 min. Then, intravascular pressure was increased to 60 mm Hg and held steady throughout the experiment to develop and maintain arterial myogenic tone. Drugs were dissolved to make stock solutions, diluted in PSS to final concentration, and applied to the artery via chamber perfusion. The effect of drug applications was evaluated at the time it reached a maximal, steady level. For experiments with de-endothelized arteries, endothelium was removed by passing an air bubble into the vessel lumen for 90 sec prior to vessel cannulation. This method is highly effective for removing the endothelial layer. The absence of a functional endothelium was confirmed by the absence of
vasodilation in response to an endothelium-dependent vasodilator (10 μM acetylcholine) while vasodilation in response to an endothelium-independent vasodilator (10 μM sodium nitroprusside) was observed.¹²

**Cholesterol and protein determination in de-endothelized arteries.** Middle cerebral arteries from adult male Sprague-Dawley rats (≈250 g) were dissected, cannulated, and de-endothelized as described in the previous section. De-endothelized arteries from each animal were incubated in PSS or PSS containing either MβCD (5 mM) or MβCD+CLR complex (8:1 molar ratio) at 37°C for 1 h. After this treatment, arteries from each treatment group were centrifuged at 14,000 rpm and 4°C for 5 min. The supernatant was discarded, and the pellet was resuspended in 300 μL of phosphate-buffered saline (PBS) (Gibco) containing 1% Triton X-100 (Fisher Scientific). The pellet was homogenized with a Dounce homogenizer and sonicated for 15-20 s three times every 15 s. Sonication was performed on ice, using Sonic Dismembrator (Fisher Scientific). The homogenized pellet was centrifuged at 14,000 rpm and 4°C for 2 min, the homogenate being collected and stored at -80°C until protein or cholesterol determinations. Protein levels were determined using the Pierce BCA protein assay kit (Thermo Scientific) according to the manufacturer’s protocol. For colorimetric readings, all standards and samples were placed in 96-well flat bottom maxi-sorp microplates (Nunc, Inc.). Data acquisition and analysis were conducted using the Bio-Tek plate reader and KC4 software (Bio-Tek Instr., Inc.) at a wavelength of 562 nm.

Cholesterol determinations were made using the Amplex Red Cholesterol Assay kit (Molecular Probes, Inc.) following the manufacturer’s specifications. Standards and samples were placed in 96-well flat bottom maxi-sorp microplates (Nunc, Inc.) for fluorescence readings with excitation and emission set at 530-560 nm and 590 nm, respectively. Data acquisition and analysis were performed with Bio-Tek plate reader and KC4 software (Bio-Tek Instruments, Inc.).
**Isolation of arterial myocytes from rat and mouse.** Basilar and middle cerebral arteries were dissected out from each brain under a stereo-zoom microscope (Nikon C-PS) and placed into ice-cold dissociation medium (DM) with the following composition (mM): 0.16 CaCl₂, 0.49 EDTA, 10 HEPES, 5 KCl, 0.5 KH₂PO₄, 2 MgCl₂, 110 NaCl, 0.5 NaH₂PO₄, 10 NaHCO₃, 0.02 phenol red, 10 taurine, 10 glucose. Each artery was cut into 1- to 2-mm long rings (up to 30 rings/experiment). Individual myocytes were enzymatically isolated following a procedure described in detail elsewhere,³ with slight modifications. Briefly, rat arterial rings were put in 3 ml DM containing 0.03% papain, 0.05% bovine serum albumin (BSA), and 0.004% dithiothreitol at 37°C for 15 min in a polypropylene tube and incubated in a shaking water bath at 37°C and 60 oscillations/min for 15 min. Then, the supernatant was discarded and the tissue transferred to a polypropylene tube with 3 ml DM containing 0.06% soybean trypsin inhibitor, 0.05% BSA, and 2% collagenase (26.6 units/ml). The tube was incubated again in a shaking water bath at 37°C and 60 oscillations/min for 15 min. Mouse arterial rings were initially put in 3 ml DM containing 0.00075% papain, 0.05% BSA, and 0.004% dithiothreitol at 37°C for 12 min in a shaking water bath at 60 oscillations/min. Then the tissue was transferred to 3 ml DM containing 0.06% soybean trypsin inhibitor, 0.05% BSA, and 2% collagenase (26.6 units/ml) and incubated under similar conditions for 10 min. Finally, either rat or mouse artery tissue pellet was transferred into a tube with 3 ml of DM containing 0.06% soybean trypsin inhibitor. Tissue-containing DM was pipetted using a series of borosilicate Pasteur pipettes having fire-polished, diminishing internal diameter tips. The procedure rendered a cell suspension containing relaxed, individual myocytes (≥5 myocytes/field using a 20X objective) that could be identified under an Olympus IX-70 microscope (Olympus America). The cell suspension was stored in ice-cold DM containing 0.06% BSA, and the cells were used for patch-clamp recordings up to 3 h after isolation.

**Electrophysiology experiments on freshly isolated rat/mouse arterial myocytes.** BK currents at single channel resolution were recorded from excised, I/O membrane patches. For experiments
with rat myocytes, both bath and electrode solutions contained (mM) 130 KCl, 5 EGTA, 2.28 MgCl₂, 15 HEPES, 1.6 HEDTA, 5.22 CaCl₂ ([Ca²⁺]ₘₐₓ=10 µM), pH 7.35. For experiments with mouse myocytes, both bath and electrode solutions contained (mM) 130 KCl, 5 EGTA, 2.44 MgCl₂, 15 HEPES, 1.6 HEDTA, 5.59 CaCl₂ ([Ca²⁺]ₘₐₓ=30 µM), pH 7.35. Nominal free Ca²⁺ was calculated with MaxChelator Sliders (C. Patton, Stanford University, CA) and validated experimentally using Ca²⁺-selective electrodes (Corning Incorporated Science Products Division).

Patch-recording glass electrodes were made as described. Immediately before recording, the tip of each electrode was fire-polished on a microforge WPI MF-200 (World Precision Instruments) to give resistances of 5-9 MΩ when filled with electrode solution. An agar bridge with Cl⁻ as the main anion was used as ground electrode.

Before starting patch-clamp data acquisition, freshly isolated myocytes were divided into two experimental groups: a) control, or naïve CLR content, b) cholesterol-depleting treatment. The groups of myocytes were exposed for 20 min to recording bath solution or recording bath solution containing 5 mM MβCD, respectively. Membrane patches were excised from myocytes from the two experimental groups and exposed to a stream of EtOH-free (control) or EtOH-containing (50 mM) bath solution. Solutions were applied onto the patches by using a computerized and pressurized DAD12 system (ALA Scientific Instruments) via a micropipette tip with an internal diameter of 100 µm. Experiments were carried out at room temperature (21°C). Ionic current was recorded with an EPC8 amplifier (HEKA) at 1 kHz using a low-pass, eight-pole Bessel filter (model 902LPF; Frequency Devices). Data were digitized at 5 kHz using Digidata 1320A and pCLAMP 8.0 (Molecular Devices).

**Bilayer experiments.** Using Lipofectamin 2000 (Invitrogen), HEK293 cells were transiently transfected with cDNAs encoding BK channel-forming (cbv1, AY330293) and accessory (β1, FJ154955) subunits cloned from rat cerebral artery myocytes. Two days after transfection, cells were harvested, pelleted, resuspended, placed on ice, and sonicated with 10 ml of buffer solution.
(mM): 30 KCl, 2 MgCl₂, 10 HEPES, 5 EGTA, pH 7.2. Sonication was performed twice for 15 s at 1 min interval. A partially purified membrane preparation was obtained using a sucrose gradient as previously described,⁹ and aliquots were stored at -80°C.

Cbv1 channels were incorporated by adding 3-5 µl of membrane preparation onto bilayer cast of POPE:POPS, 3:1 (w/w). Cholesterol was dissolved in chloroform and then introduced into the lipid mixture at a final concentration of 23 mol%. The lipid mixture was dried under N₂ gas and resuspended in 25 mg/ml decane. Vertical, 80-120 pF bilayers were formed by painting the lipid mix across a 200-µm diameter hole in a delrin cup (Warner Instruments). Vesicle fusion was promoted by osmosis and stirring, with the cis chamber (to which the membrane prep was added) being hyperosmotic to the trans chamber solution. Recording solutions consisted of (mM) cis, 300 KCl, 10 HEPES, 1.47 HEDTA, 1.05 CaCl₂ (free Ca²⁺=10 µM), pH 7.2, and trans, 30 KCl, 10 HEPES, 0.1 HEDTA, pH 7.2. Nominal free Ca²⁺ in solution was calculated and validated experimentally as described above for patch-clamp recordings. The trans chamber was held at ground while the cis chamber was held at potentials relative to ground. Only channels with their intracellular Ca²⁺-sensors oriented towards the cis chamber were considered for experimentation. For evaluation of EtOH effect, 50 mM EtOH was added to the cis chamber. After EtOH addition, cis chamber was covered with parafilm to prevent EtOH evaporation. For evaluation of paxilline block of recombinant BK channels, 1 µM paxilline in bathing solution was present in the trans chamber.

Ion currents were obtained for 3-5 min of continuous recording at 0 mV using a Warner BC-525D amplifier, low-pass filtered at 5 kHz using the 4-pole Bessel filter built in the amplifier, and sampled at 35 kHz with Digidata 1322A/pCLAMP 9.2 (Molecular Devices). For a proper comparison with data previously obtained by us⁸-¹¹ and others,¹²-¹⁴ all studies were conducted at room temperature (20-25°C).

**Chemicals.** Cholesterol, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine sodium salt (POPS) were purchased from Avanti
Polar Lipids. EtOH (100% purity) was purchased from American Bioanalytical. All other chemicals were purchased from Sigma. EtOH was freshly diluted in bath solution immediately before application to the artery, myocyte, or bilayer prep. Unless otherwise specified, each pressurized artery, myocyte membrane patch, or artificial lipid bilayer was exposed to EtOH only once to avoid reduced responsiveness of BK current to multiple applications of EtOH.\textsuperscript{15}

**Data analysis.** Artery diameter data were analyzed using IonWizard 4.4 software (IonOptics). The “arterial diameter before drug application” was obtained by averaging diameter values during 3 minutes of recording immediately before drug application. A drug-induced change in arterial diameter was determined from the peak effect obtained during drug application.

Myogenic (arterial) tone was calculated according to the formula: myogenic tone (\%)=(1-active diameter/passive diameter) x 100.\textsuperscript{16}

Electrophysiological data were analyzed with Clampfit 9.2 (Molecular Devices). In patch-clamp recordings, NPo was used as index of channel steady-state activity,\textsuperscript{17-18} where:

\[
\text{NPo}=N \times \text{Po}
\]

The number of channels in the patch was determined after applying a depolarizing voltage step to +60 mV. At this voltage, and in the presence of [Ca\textsuperscript{2+}]\textsubscript{free} used in our experiments (10-30 \(\mu\)M), BK channels reach maximal activation, with Po~1. Thus, N can be clearly determined from the number of opening levels. Knowing N, NPo and Po were both obtained using the built-in option in Clampfit 9.2 (Molecular Devices) from at least 1 min of continuous recording under each experimental condition. In bilayer recordings, we used bilayers with only one channel incorporated (N=1). Thus, NPo=Po, and Po was used as an index of channel steady-state activity. Further analysis, plotting, and fitting of data were conducted using Origin 7.0 (Originlab Corp) and InStat 3.0 (GraphPad Software Inc). Gaussian distribution of data was tested using a Kolmogorov-Smirnov test.
Supplementary Discussion

BK subunits and variant gating mechanisms differentially contribute to cholesterol/ethanol individual actions on BK Po and render synergistic inhibition of Po by the two modulators. While both CLR and EtOH target cerebrovascular BK, the relative contribution of channel-forming cbv1 and accessory β1 to each modulator’s action and the underlying mechanisms clearly differ. Expanding our previous results with hSlo1 subunits from human brain\(^9\) and cbv1,\(^{11,19}\) current results demonstrate that cbv1 is sufficient to sustain CLR inhibition of BK, with BK β1 exerting no modulatory role on this CLR action under our experimental conditions (Figs. 4, 5). In a recent structure-activity study,\(^{19}\) we demonstrated that CLR inhibition of cbv1 channels required a β configuration of the hydroxyl group at C3, that is, a very strict structural requirement. Moreover, the enantiomer of natural cholesterol failed to decrease BK activity, indicating that CLR inhibition of BK is secondary to CLR recognition by a protein surface, very likely provided by the cbv1 subunit itself. Upon this recognition, CLR modulation of BK function is primarily reflected in decreased Po, which results from gating modification and, thus, changes in both open and closed time distributions.\(^{19}\) Remarkably, CLR inhibition of cbv1 channels is identical whether the channel is either gated by positive voltage or activating Ca\(^{2+}\)\(_i\),\(^{20}\) suggesting that Ca\(^{2+}\) binding to the channel is not necessary for CLR to inhibit BK activity.

As found for CLR, EtOH modulation of BK may occur in absence of BK β1 subunits.\(^{8,14}\) In sharp contrast to CLR, however, this EtOH-BK interaction requires activating Ca\(^{2+}\)\(_i\) levels: the channel-forming subunit is EtOH-sensitive only when gated by Ca\(^{2+}\)\(_i\) but not by positive voltage.\(^{18}\) Moreover, EtOH action on BK channel-forming subunits (hSlo1, cbv1) becomes a function of activating ligand: at submicromolar and tens of micromolar Ca\(^{2+}\)\(_i\), EtOH, respectively, activates and inhibits the channel,\(^{8,18}\) the latter likely via Ca\(^{2+}\)-mediated desensitization.\(^{18}\) Therefore, in contrast to CLR-, EtOH-induced inhibition of homomeric cbv1 is detected only under high, usually nonphysiological, Ca\(^{2+}\)\(_i\) conditions.
BK β1 is uniquely suited to potentiate the apparent Ca\textsuperscript{2+}-sensitivity of the channel, an action that is evident by a β1-induced leftward shift in the channel Po/Po\textsubscript{max}-voltage relationship.\textsuperscript{8,11,21} Furthermore, it is this β1-induced potentiation of the channel apparent Ca\textsuperscript{2+}-sensitivity what likely explains the EtOH-induced reduction of Po when the cerebrovascular BK is studied under physiological conditions of Ca\textsuperscript{2+} and voltage, as done in the current and previous studies.\textsuperscript{1,8} In synthesis, both subunit relative contribution and gating mechanisms involved in CLR vs. EtOH inhibition of BK differ, each leading to decrease Po and synergistic inhibition of channel function and cerebral artery constriction.
Supplementary Figure Legends

Supplementary Figure I. Manipulation of cholesterol levels by MβCD or MβCD+CLR complex treatment after myogenic tone development fail to alter cerebral artery tone. (A) Myogenic tone in rat middle cerebral arteries after CLR depletion with 5 mM MβCD or CLR enrichment with 5 mM MβCD+0.625 mM CLR (see Materials and Methods) is similar to that in arteries with intact CLR content (naïve CLR). (B) Average changes in arterial diameter in the presence of 5 mM MβCD or 5 mM MβCD+ 0.625 mM CLR. For both A and B panels: (n)=number of arteries; each artery was dissected from a different brain.

Supplementary Figure II. Average arterial tone and diameter changes in response to cholesterol-manipulating treatment of endothelium-free middle cerebral arteries. (A) Myogenic tone in arteries exposed to 5 mM MβCD (CLR depletion) or 5 mM MβCD+0.625 mM CLR (CLR enrichment) is similar to that from arteries with naïve CLR content. (B). Average changes in arterial diameter in presence of 5 mM MβCD or 5mM MβCD+ 0.625 mM CLR. In both panels: (n)=number of arteries. Each artery was dissected from different brain.

Supplementary Figure III. Ethanol-induced cerebral artery constriction is also modulated when the drug is applied in the presence of MβCD or MβCD+CLR. (A) Arterial responses to KCl and EtOH before (KCl I, EtOH I) and after (KCl II, EtOH II) CLR depletion (MβCD) (B) or enrichment (MβCD+CLR) (C). (D) Averaged change in arterial diameter in response to first (I) and second (II) KCl or EtOH applications. †Different from EtOH II tested on the artery with naïve CLR level (P<0.05). (E) Averaged change in arterial diameter in response to first (I) and second (II) KCl or EtOH applications. *Different from arteries with naïve CLR level (P<0.05); (n)=number of arteries.
Supplementary Figure IV. Cbv1+β1 BK complexes in cholesterol-free (CLR-free) bilayers retain sensitivity to paxilline block. (A) Following channel reconstitution into CLR-free POPE:POPS (3:1 w/w) bilayer, 1 μM paxilline drastically decreased cbv1+β1 activity (Po) from its pre-paxilline values. Channel openings=upward deflections, arrows=baseline. V_m= 0 mV; free Ca^{2+}≈10 μM. (B) Average cbv1+β1 channel Po in the absence and presence of paxilline.
***Different from pre-paxilline Po (P<0.0001); (n)=number of bilayers.

Supplementary Figure V. BK channel open probability in wt (C57BL/6) and KCNMB1 K/O mouse cerebral artery myocytes with naïve vs. cholesterol-depleted membrane content. (A) BK open channel probability (Po) obtained at V_m=-20mV and free Ca^{2+}=30 μM from I/O membrane patches excised from C57BL/6 mouse cerebral artery myocytes with naïve vs. depleted CLR levels. (B) BK open channel probability (Po) obtained at V_m=-20mV and free Ca^{2+}=30 μM from I/O membrane patches excised from KCNMB1 K/O mouse cerebral artery myocytes with naïve vs. those from myocytes with naïve CLR content. In both panels: (n)=number of membrane patches tested. Each patch was excised from a different myocyte.

Supplementary Figure VI. Average myogenic tone and changes in artery diameter in response to cholesterol-depletion of pressurized cerebral arteries from C57BL/6 vs. KCNMB1 K/O mouse. (A). Myogenic tone of cerebral arteries with naïve CLR content or after treatment with 5 mM MβCD (CLR depletion) is similar in both C57BL/6 and KCNMB1 K/O mice. (B) Average changes in artery diameter in the presence of 5 mM MβCD. In both panels: (n)=number of arteries. Each artery was dissected from different brain.
Supplementary References


Suppl. Fig. I

**A**

Intact endothelium

- **Naive CLR**
- **CLR depletion**
- **CLR enrichment**

Myogenic tone (%)

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<th>Naive CLR</th>
<th>CLR depletion</th>
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<tr>
<td>Myogenic tone</td>
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<td>16</td>
<td>16</td>
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**B**

Intact endothelium

- **M\(\beta\)CD**
- **M\(\beta\)CD + CLR**

Change in arterial diameter (% of diameter before drug application)

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<td>Change in diameter</td>
<td>-5</td>
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Suppl. Fig. II
Suppl. Fig. III
Suppl. Fig. IV

A

Cbv1+β1

CLR-free/pre-Paxilline  Po=0.91

CLR-free/Paxilline (10 min)  Po=0.11

0.8 s  5 pA

B

(4)  ***  (4)

P0

pre-Paxilline  Paxilline

Suppl. Fig. IV
Suppl. Fig. V

A

Wild type (wt, C57BL/6)

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<td>Po</td>
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B

KCNMB1 K/O

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<tbody>
<tr>
<td>Po</td>
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Note: Po values represent the probability of a certain event occurring in the context of CLR depletion or lack thereof.
Suppl. Fig. VI

**A**

Wild type (C57BL/6)  
KCNMB1 K/O

<table>
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<tr>
<td>Myogenic tone (%)</td>
<td>15</td>
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**B**

Wild type (C57BL/6)  
KCNMB1 K/O

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<tr>
<td>Change in arterial diameter by McCD (%)</td>
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(3) (4) (3) (4)