Published in final edited form as:

Pflugers Arch. 2017 February ; 469(2): 263–277. doi:10.1007/s00424-016-1929-z.

DIFFERENTIAL DISTRIBUTION AND FUNCTIONAL IMPACT OF BK CHANNEL BETA1 SUBUNITS ACROSS MESENTERIC, CORONARY AND DIFFERENT CEREBRAL ARTERIES OF THE RAT

Guruprasad Kuntamallappanavar, Shivantika Bisen, Anna N. Bukiya, and Alex M. Dopico

Department of Pharmacology, College of Medicine, The University of Tennessee Health Science Center, Memphis, TN 38103

Abstract

Large conductance, Ca^{2+}_i - and voltage-gated K^+ (BK) channels regulate myogenic tone and thus, arterial diameter. In smooth muscle (SM), BK channels include channel-forming α and auxiliary $\beta 1$ subunits. BK $\beta 1$ increases the channel's Ca^{2+} sensitivity allowing BK channels to negatively feed-back on depolarization-induced Ca^{2+} -entry, oppose SM contraction and favor vasodilation. Thus, endothelial-independent vasodilation can be evoked through targeting of SM BK $\beta 1$ by endogenous ligands, including lithocholate (LCA). Here, we investigated the expression of BK $\beta 1$ across arteries of the cerebral and peripheral circulations, and the contribution of such expression to channel function and BK $\beta 1$ -mediated vasodilation. Data demonstrate that endothelium-independent, BK $\beta 1$ -mediated vasodilation by LCA is larger in coronary (CA) and basilar (BA) arteries than in anterior cerebral (ACA), middle cerebral (MCA), posterior cerebral (PCA), and mesenteric (MA) arteries, all arterial segments having a similar diameter. Thus, differential dilation occurs in extracranial arteries which are subjected to similar vascular pressure (CA vs. MA), and in arteries that irrigate different brain regions (BA vs. ACA, MCA, PCA). SM BK channels from BA and CA displayed increased basal activity and LCA responses, indicating increased BK $\beta 1$ functional presence. Indeed, in the absence of detectable changes in BK α , BA and CA myocytes showed an increased location of BK $\beta 1$ in the plasmalemma/subplasmalemma. Moreover, these myocytes distinctly showed increased BK $\beta 1$ mRNA levels. Supporting a major role of enhanced BK $\beta 1$ transcripts in artery dilation, LCA-induced dilation of MCA transfected with BK $\beta 1$ cDNA was as high as LCA-induced dilation of untransfected BA or CA.

Keywords

MaxiK channel; BK $\beta 1$ subunit; *KCNMB1*; cerebral artery myocyte; vasodilation; vascular smooth muscle; lithocholate

Introduction

Association of membrane spanning, ion channel-forming proteins with accessory subunits is an almost universal feature in membrane biology. Accessory subunits, while not contributing to ion channel pore formation itself, may drastically contribute to permeation, gating or the pharmacological properties of the resulting channel protein heteromer. Thus, in most mammalian tissues, voltage- and Ca^{2+} -gated, large conductance K^+ (BK) channels result from tetrameric association of channel-forming α subunits (encoded by the *KCNMA1* or *Slo1* gene) and regulatory β subunits [71]. These regulatory subunits are small proteins characterized by a common structural design: two transmembrane domains (TM1 and TM2) connected by an extracellular loop (ECL) that end in two short termini (N- and C-ends) [11,12,47,85,87,90]. Four types of BK β subunits have been identified, each encoded by different genes (*KCNMB1-4*). Remarkably, *KCNMB* expression is rather tissue-specific: BK $\beta 1$ is highly abundant in smooth muscle whereas BK $\beta 4$ is widely distributed across central neurons [7,11,47,87]. This differential expression raises the possibility that modification of BK channel phenotype by BK β s is set to regulate physiology in a tissue-specific manner.

In vascular smooth muscle, association of BK $\beta 1$ with *slo1* increases the channel's apparent Ca^{2+} -sensitivity, which allows activation of BK channels at physiological negative voltages. Upon activation, BK channels generate outward K^+ currents that counteract depolarization-induced Ca^{2+} -entry and thus oppose cell contraction while favoring smooth muscle relaxation and eventual vasodilation [44,52]. Moreover, BK $\beta 1$ have been identified as critical transducers between BK channel activation and " Ca^{2+} -sparks", a local Ca^{2+} signal generated by the activity of sarcoplasmic reticulum ryanodine receptors (RyR) [44,52], with Ca^{2+} -spark-BK channel coupling *via* BK $\beta 1$ leading to vasodilation [12]. The rather selective expression of BK $\beta 1$ in smooth muscle and the role of this subunit in regulating smooth muscle tone and vascular diameter has led to increasing efforts to determine whether important physiological signals (e.g., PIP2, leukotriene B4, estradiol and other steroids) increase BK channel activity in smooth muscle and thus, evoke vasodilation by interacting with BK $\beta 1$ subunits (reviewed in [30]). Our laboratory has demonstrated that the bile acid lithocholate (LCA) at μM levels causes artery dilation by smooth muscle BK channel activation. Moreover, this LCA action is abolished following genetic ablation of BK $\beta 1$ subunits [15]. Remarkably, all other known β subunits (i.e., $\beta 2$, $\beta 3$ and $\beta 4$) are unable to substitute for $\beta 1$ in endowing the BK channel with sensitivity to μM LCA [20]. Thus, μM LCA has become a powerful pharmacological tool to test the functional presence of BK $\beta 1$ subunits and BK $\beta 1$ subunit-mediated, endothelium-independent vasodilation [15,16,20,55].

A differential functional expression of BK $\beta 1$ across different arterial nets may lead one vascular territory and the organ/region under its perfusion to become more susceptible to modulation by endogenous signals and thus, participate in a more or lesser degree in vascular components of pathophysiological processes (e.g., inflammation involving LTB4 or hyperdynamic states associated with porto-systemic shunt of bile acids, including LCA). Likewise, such differential expression may render some vascular nets more sensitive to exogenous vasodilators that act *via* selective targeting of smooth muscle BK $\beta 1$ subunits (e.g., HENA; [16]). In this study, we used quantitative real-time PCR, confocal fluorescence

microscopy, patch-clamp electrophysiology, *in vitro* electroporation, Western blotting, artery *in vitro* pressurization and diameter monitoring in response to an endogenous BK $\beta 1$ ligand (LCA) to demonstrate for the first time a differential functional expression of BK $\beta 1$ in smooth muscle complemented with a differential response to LCA (a) between cerebral and peripheral systemic arteries (i.e., quaternary branches of MA and secondary branches of BA); (b) in arteries within the systemic circulation (quaternary branches of MA vs. CA), and (c) in cerebral arteries that provide blood flow to different brain regions (ACA, MCA and PCA vs. BA). Our study reveals that CA and BA arteries have the highest functional expression of BK $\beta 1$, making them more susceptible to BK $\beta 1$ -mediated dilation. Moreover, we could enhance the dilation of arteries that have naturally low BK $\beta 1$ function (i.e., middle cerebral artery, MCA) by enriching the artery with the *KCNMB1*-coding cDNA. Our data provide a molecular basis for the differential endothelium-independent dilation of arterial territories that irrigate different peripheral and brain targets, with practical consequences for vasodilating therapy.

Material and Methods

Pressurized artery diameter measurements

Adult male Sprague-Dawley rats (≈ 250 g) were decapitated using a guillotine. This procedure was approved by the Institutional Animal Care and Use Committee from the University of Tennessee Health Science Center (UTHSC), an AAALAC accredited institution. Rat ACA, MCA, PCA, BA (secondary branches), MA (quaternary branches), and CA were dissected on ice under a Nikon SMZ645 microscope (Nikon C-PS, Tokyo, Japan) and cut into 1 to 2 mm-long segments. A segment was cannulated at each end in a temperature-controlled, custom-made perfusion chamber. Arteries were de-endothelialized 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 μ M acetylcholine) while vasodilation in response to an endothelium-independent vasodilator (10 μ M sodium nitroprusside) was preserved [13–15,18,59].

Using a Dynamax RP-1 peristaltic pump (Rainin Instr., Columbus, OH), the chamber was continuously perfused at a rate of 3.75 ml/min with PSS (mM): 119 NaCl, 4.7 KCl, 1.2 KH_2PO_4 , 1.6 CaCl_2 , 1.2 MgSO_4 , 0.023 EDTA, 11 glucose, 24 NaHCO_3 . PSS was equilibrated at pH 7.4 with a 21/5/74% mix of $\text{O}_2/\text{CO}_2/\text{N}_2$ and maintained at 35–37°C. Arteries were monitored with a CCD camera (Sanyo VCB-3512T; Sanyo, Osaka Prefecture, Japan) attached to an inverted microscope (Nikon Eclipse TS100; Nikon, Tokyo, Japan). As routinely done by us [13,15,16,18,22,59] and others [1,4,6,10,34,35,81], the artery external wall diameter (ED) was measured using the automatic edge-detection function of IonWizard software (IonOptics, Westwood, MA) and digitized at 1 Hz. 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 Instrumentation, St Albans City, VT). 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 allow arteries to develop and maintain 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 application on artery ED was evaluated at the time ED reached a maximal, steady level.

Isolation of rat artery myocytes

Individual myocytes were enzymatically isolated from ACA, MCA, PCA, BA, MA or CA arterial rings (see above) following a procedure described in detail elsewhere [14,18,31,59]. 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, Center Valley, PA). The cell suspension was stored in ice and the cells were used for patch-clamp recordings and fluorescence labeling up to 3 h after isolation.

Electrophysiology experiments on BK channels in arterial smooth muscle cells

To measure the basal activity of smooth muscle BK channels in arteries from different territories, single-channel BK currents were recorded from excised, inside-out (I/O) membrane patches at 3 μM free Ca^{2+}_i over a wide voltage range (-50 mV to $+100$ mV). For studies involving application of 45 μM LCA, single channel recordings were obtained at $V_m = -40$ mV. Lithocholate was applied to the intracellular side of I/O patches. For LCA experiments with rat myocytes, $[\text{Ca}^{2+}_i]$ free was set at 3 μM .

For patch-clamp experiments, both bath and electrode solutions contained (mM) 130 KCl, 5 EGTA, 2.28 MgCl_2 , 15 HEPES, 1.6 HEDTA, 5.22 CaCl_2 ($[\text{Ca}^{2+}]$ free ≈ 3 μM), pH 7.35. Nominal free $[\text{Ca}^{2+}_i]$ was calculated with MaxChelator Sliders (C. Patton, Stanford University, CA) and validated experimentally using Ca^{2+}_i -selective electrodes (Corning Incorporated Science, Corning, NY) [28]. Patch-recording glass electrodes were prepared as described elsewhere [29]. Immediately before recording, the tip of each electrode was fire-polished on a WPI MF-200 microforge (World Precision Instruments, Sarasota, FL) to give resistances of 2–5 M Ω when filled with electrode solution. An agar bridge with Cl^- as the main anion was used as ground electrode. Data were acquired using an EPC8 amplifier (HEKA Electronics, Lambrecht, Germany), low-passed at 1 kHz using an eight-pole Bessel filter (Frequency Devices, Ottawa, IL), and digitized at 5 kHz using a Digidata 1320A A/D converter and pCLAMP 8.0 (Molecular Devices, Sunnyvale, CA).

After excision of the membrane patch from the cell, the patch was consecutively exposed to a stream of bath solution containing 0.1% (v/v) DMSO (i.e., control solution), then bath solution containing 45 μM LCA followed by the patch exposure to control solution to wash LCA out. Solutions were applied onto the patches using a computerized and pressurized DAD12 system (ALA Scientific Instruments, Farmingdale, NY) *via* a micropipette tip with an internal diameter of 100 μm . Experiments were carried out at room temperature (21°C).

Immunocytochemistry and confocal fluorescence imaging

Freshly dispersed myocytes were first allowed to settle/attach on a coverslip on ice for 30 min. Then, they were fixed in 4% paraformaldehyde for 10 min. Fixed myocytes were

permeabilized with 0.1% Triton-100 for 10 min and then incubated with blocking solution containing 20% goat serum and 2% bovine serum albumin for 30 min. The cover slips were then incubated with mouse monoclonal anti-slo1 subunit antibody (Neuromab, UC Davis) and rabbit polyclonal anti-BK β 1 subunit antibody (Thermo Scientific, Waltham, MA) at 4°C overnight. Then, cells were washed with PBS and incubated with pre-absorbed Alexa-Fluor 488-conjugated anti-mouse and Cy5-conjugated anti-rabbit secondary antibodies at room temperature in the dark for 2 hr. Cover slips were mounted on slides using ProLong AntiFade kit (Invitrogen, Carlsbad, CA) and sealed using clear nail polish. Immunofluorescence images were obtained sequentially using the 488 and 635 laser lines of an Olympus FV-1000 laser scanning confocal system (Olympus American Inc., Center Valley, PA). Laser power, photomultiplier tube gain, and offset parameters were identical in all experiments.

Western blotting

Western blotting was performed as described in detail elsewhere [50,51].

Quantitation of BK α and β 1 subunit mRNAs in rat arteries

Total RNA was extracted from de-endothelialized rat arteries using TRIzol (Life Technologies, Carlsbad, CA, USA) as described elsewhere [25]. Reverse transcription was conducted from 1 μ g of total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) following the manufacturer's instructions. BK β 1 transcript levels were determined by quantitative real-time PCR. Quantitative real-time PCR was performed in 96-well plates with a 480 LCycler (Roche, Basel, Switzerland) using the LC480 Master Mix and a Universal Probe Library (UPL) probe (Roche, Basel, Switzerland) at a concentration of 10 μ M in a final reaction volume of 10 μ l under the following conditions: 95°C for 5 min for activation and 45 cycles of 95°C for 10 s, 60°C for 60 s, and 72°C for 10 s for amplification. Negative controls without cDNA were run for each reaction. After testing six endogenous control genes, Tata Binding Protein (TBP) was used as an endogenous control gene. Sequences of primers and relevant probes for BK α , β 1 and TBP are shown in Table 1. All samples were analyzed in triplicates. Relative mRNA levels were normalized to TBP mRNA levels obtained from the same sample of cDNA. Expression of BK α and β 1 relative to TBP in each sample was calculated on the basis of the C_t method (where C_t is threshold cycle), as previously described [60]. Gene specific primers and probes were designed using the Universal Probe Library (UPL; <http://www.roche-applied-science.com/sis/rtPCR/upl/index.jsp>). These experiments were conducted at the UTHSC Molecular Resource Center.

Electroporation of cerebral arteries

In order to overexpress BK β 1 in MCA, these arteries were placed into an electroporation chamber (Pulse generator model CUY21 Vitro-Ex; Bex, Co., Ltd, Itabashiku, Tokyo) at room temperature in Ca^{2+} -free phosphate buffered saline (PBS) containing 15 μ g of either empty vector (pcDNA3) or pcNA3- β 1 (pcDNA3-containing the β 1 insert). Tandem pulse electroporation was applied to the arteries. Then, arteries were removed from the chamber and maintained in PBS containing either empty vector or BK- β 1 for 10–15 min before being placed in PBS for an additional 15 min. The transfected arteries were then placed in

DMEM-F12 media (serum-free) supplemented with 1% penicillin–streptomycin (Sigma-Aldrich, St. Louis, MO) for 3–4 days under standard conditions (5% CO₂; 37°C). Arteries transfected with either empty vector or BK β 1 were then used for experimentation: Western blotting or pressurized artery diameter measurement.

Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma Aldrich (St. Louis, MO). Just before the experiment, LCA was initially dissolved in DMSO to obtain a stock solution of 33 mM. Stock solution was sonicated for 30 min. Then, to obtain final LCA concentration (45 μ M), the stock solution was further diluted with PSS.

Data analysis

Artery diameter data were analyzed using IonWizard 4.4 software (IonOptics, Westwood, MA). The “arterial diameter before drug application” was obtained by averaging ED values recorded for 5 min immediately before drug application. A drug-induced change in arterial ED was determined from the peak effect obtained during drug application. For patch clamp recordings at single channel resolution, the product of the number of channels in the patch (N) and the channel individual open probability (P_o) was used as an index of channel steady-state activity. Using a built-in function in Clampfit 9.2 (Molecular Devices), NP_o was calculated from at least 30 secs of gap-free recordings under each experimental condition.

Fluorescence was quantified using built-in function in FV10-ASW 3.1 software (Olympus American Inc., Center Valley, PA) as described [13]. The fluorescence corresponding to the plasmalemma-subplasmalemma region was obtained following direct superposition of the myocyte fluorescent image with the corresponding image of the myocyte under visible light. Fluorescence was quantified by measuring the intensity of pixels above a set threshold defined as the mean fluorescence intensity outside the cells (i.e., background).

Data are expressed as mean $\pm$ SEM (n=number of patches/arteries), and plotted and fitted using Origin 8.5 (Originlab, Northampton, MA) and InStat3 (GraphPad, La Jolla, CA).

Results

Effect of *in vitro* lithocholate exposure on diameter from de-endothelialized cerebral, mesenteric and coronary arteries

Our group has previously reported that LCA dilates rat MCA in an endothelium-independent fashion [15,16]. Thus, we first determined the effect of 45 μ M LCA on de-endothelialized ACA, PCA, BA, MA and CA, using de-endothelialized MCA as our “positive control”. The LCA concentration chosen constitutes EC₅₀ for LCA to activate smooth muscle BK channels and thus increase MCA diameter [15]. This LCA concentration can also be found in systemic blood following spillover from the portal to the systemic circulation under several pathological conditions [37,41,56,82]. After development of myogenic tone, maximal artery contraction was determined by perfusing the vessel with 60 mM KCl. At the end of each experiment, the artery was exposed to a Ca²⁺-free solution for determination of passive diameter (Fig. 1a). Treatment with control (DMSO-containing) solution

(composition in Material and Methods) did not produce significant change in the diameter of any of the arteries tested, as representatively shown with MCA (Fig. 1a). In contrast, 45 μ M LCA increased ED in all cases ($p<0.05$), averaging 3.5 ± 0.46 , 4.59 ± 0.8 , 4.6 ± 1.08 , 12.9 ± 1.8 , 4.4 ± 0.6 and $10.4\pm0.5\%$ increase over pre-LCA values for ACA, MCA, PCA, BA, MA and CA, respectively (Fig. 1b). These results document a significantly different response to LCA from arteries that 1) belong to cerebral vs. systemic circulation (e.g., MCA vs. CA); 2) belong to systemic circulation (e.g., CA vs. MA), and 3) branch out from the Willis' Circle itself (e.g., ACA/MCA/PCA vs. BA). Our data were obtained in de-endothelialized arteries under continuous bath perfusion, which results in the wash out of freely diffusible signaling molecules. Moreover, smooth muscle accounts for 52% of the total vessel wall in cerebral arteries [53]. Thus, it is possible to hypothesize that the differential endothelium-independent vasodilation evoked by LCA on the different arteries probed in our study is mediated by targets and mechanisms that operate within the vascular smooth muscle myocyte.

BK channels in myocytes from basilar and coronary artery are highly sensitive to lithocholate

Previous studies [15] on rat MCA demonstrate that LCA-induced artery dilation is due to steroid action on the arterial myocyte itself. Furthermore, we found LCA-mediated MCA dilation was mediated by LCA activation of vascular smooth muscle BK channels [15]. Thus, we hypothesize that vascular smooth muscle BK channels isolated from arteries that presented a differential vasodilating response to LCA (Fig. 1) are differentially activated by LCA at concentrations that evoke vasodilation. To test this hypothesis, we evaluated LCA action on BK channel steady-state activity (i.e., the product $N\times P_o$, see Material and Methods) by using I/O patch-clamp recordings in myocytes freshly isolated from ACA, MCA, PCA, BA, MA and CA. In all cases, the membrane potential was set to -40 mV and free $[Ca^{2+}]_i$ to 3 μ M: these conditions reproduce the transmembrane voltage and intracellular Ca^{2+}_i level found in the vicinity of the BK channel during myocyte contraction, that is, when BK channels exert a physiological negative feed-back on depolarization-induced arterial myocyte contraction [48,76]. Paralleling the drug application protocol used with the arterial segments in artery diameter determination experiments (see above), each excised patch was sequentially exposed to vehicle-containing (control), LCA-containing (45 μ M final concentration) and vehicle-containing solution (LCA washout) (Fig. 2a–f). Application of LCA reversibly increased BK NP_o by 3.5 ± 1.1 , 3.6 ± 1.1 , 5.5 ± 1.6 and 3.5 ± 1.1 times of pre-LCA levels in myocyte membrane patches from ACA, MCA, PCA and MA arteries, respectively (Figs. 2 and 3a) ($p<0.05$). Remarkably, LCA increased BK NP_o approximately 14.01 ± 2.7 and 11 ± 1.9 times when compared to pre-LCA values in myocytes from BA and CA, respectively (Figs. 2 and 3a) ($p<0.05$). This LCA action is significantly larger than LCA-induced BK channel activation in ACA, MCA, PCA and MA myocytes ($p<0.05$). Moreover, the overall differential sensitivity to LCA of smooth muscle BK channels across the arteries under study highly correlates with the sensitivity of the different arteries to LCA-induced, endothelium-independent vasodilation (Pearson's $r=0.9898$; degrees of freedom=4; Fig. 3b). This extremely high positive correlation, together with previous data establishing that LCA-induced dilation of MCA is due to LCA activation of smooth muscle BK channels [15], strongly suggest that the larger LCA-induced BK channel activation in myocytes of BA

or CA *vs.* that of ACA, MCA, PCA or MA, is an important contributor to the larger LCA-induced dilation of the former group of arteries *vs.* the latter.

BK channel steady-state activity is higher in myocytes from basilar and coronary arteries

Lithocholate-induced dilation of MCA is mediated by the activation of smooth muscle BK channels *via* direct and selective LCA- $\beta 1$ subunit interaction [15,17,19]. On the other hand, it is widely accepted that within physiological levels of Ca^{2+}_i and voltage, over-expression of $\beta 1$ subunits with slo1 in heterologous systems (HEK293 cells, *X. laevis* oocytes) leads to increased BK steady-state activity [5,11,27,50,51]. The stoichiometry of slo1 and $\beta 1$ subunits in native channels across different vascular and nonvascular tissues remains largely unknown. Conceivably, in some arterial myocytes $\beta 1$ subunits do not saturate their BK channel-forming slo1 counterparts. Thus, the differential LCA sensitivity of BK channels across the different arteries may be the result of heterogeneous channel composition, i.e., the more sensitive channels may reside in arterial myocytes that have higher $\beta 1$ expression. It is also possible, however, that a different proteolipid composition across different arteries leads to differential functional coupling between slo1 and $\beta 1$ (see Discussion). This, in turn, could contribute to a regionally distinct BK channel-LCA interaction, which leads to a differential channel response to the steroid. In either case, the resulting end-point would be an increased *basal* (i.e., before LCA application) BK activity in the arterial myocytes that display the greater sensitivity to LCA. Thus, we next hypothesized that myocytes from BA and CA, which displayed increased responses to LCA when compared to their ACA, MCA, PCA and MA counterparts (Figs. 2 and 3), possessed an enhanced $\beta 1$ -driven BK function. Several studies have demonstrated that $\beta 1$ subunit-induced modification of BK channel gating at physiological conditions of voltage and Ca^{2+}_i translates into an increased basal BK NP_o [11,27,65–68]. Thus, we next compared the basal BK NP_o in myocytes isolated from ACA, MCA, PCA, MA and CA.

BK steady-state activity (given as $\text{NP}_o/\text{NP}_{o\text{ max}}$) in myocytes from the arteries under study was evaluated at $3\text{ }\mu\text{M Ca}^{2+}_i$, over a wide range of voltages (-50 to $+100\text{mV}$). Thus, from $\text{NP}_o/\text{NP}_{o\text{ max}}\text{-V}$ plots (Fig. 4a), the following V_{half} values were obtained: 58.1 ± 11 , 50.7 ± 8.6 , 65.2 ± 7.5 , 23.5 ± 5.9 , 58.98 ± 10.16 and $25.15\pm 3.4\text{ mV}$ for ACA, MCA, PCA, BA, MA, and CA, respectively (Fig. 4b). These data clearly demonstrate that BK channel steady-state activity in myocytes from BA and CA is significantly higher ($P<0.05$) than BK activity in myocytes from other arteries. Furthermore, the parallel leftward shift in the $\text{NP}_o/\text{NP}_{o\text{ max}}\text{-V}$ curve seen at physiological Ca^{2+}_i is consistent with an increased presence of functional BK $\beta 1$ subunits [5,11,27,50,51,67,70]. Interestingly, the actual BK V_{half} values in myocytes from BA and CA are similar to those found using heterologous expression systems in which overexpression of $\beta 1$ subunits is considered to saturate their slo1 partners, including slo1 cloned from rat cerebral artery myocytes ([5,11,27,50,51,70]; e.g., at physiological $3\text{ }\mu\text{M Ca}^{2+}_i$, V_{half} values for BA, CA, and slo1+ $\beta 1$ overexpressed in oocytes are 23.5 ± 5.9 , 25.15 ± 3.4 and 24.7 ± 13.0 [27]), respectively. Collectively, our electrophysiological data strongly suggest that the increased activity of myocyte BK channels in CA and BA is primarily due to an increased functional presence of BK $\beta 1$ in these arteries.

Cell surface levels of BK $\beta 1$ protein are higher in basilar and coronary artery smooth muscle cells

Although BK channels have been found in internal organelles, such as mitochondria and the nuclear envelope [57,79,80], BK channels *located in the plasmalemma* primarily control myogenic tone and, most likely, its modulation by LCA [15,75,76]. Therefore, the simplest explanation for increased myocyte BK function and vasodilation *via* $\beta 1$ subunits in BA and CA is that these modulatory proteins are expressed in the plasma membrane of myocytes in BA and CA at levels significantly higher than those found in myocytes from ACA, MCA, PCA and MA. To test this, we first used immunofluorescence staining of BK α and $\beta 1$ subunits in myocytes freshly isolated from ACA, MCA, PCA, BA, MA and CA. Data failed to reveal significant differences in BK α subunit-associated fluorescence across the different arteries (Figs. 5a and 6a). In contrast, BK $\beta 1$ subunit-associated fluorescence was significantly increased in myocytes from BA and CA when compared to that from ACA, MCA, PCA or MA (Figs. 5b and 6b). Conceivably, an increased surface presence of BK $\beta 1$ protein in myocytes from BA and CA is a main determinant of the hyperpolarized V_{half} values characteristic of BK current in myocytes from these arteries (Fig. 4b), as well as the increased BK channel activation and arterial dilation in response to LCA, a BK $\beta 1$ -ligand (see Discussion).

KCNMB1 gene expression studies in different vascular territories

To begin to explore the mechanisms underlying a differential membrane surface expression of BK $\beta 1$ proteins in myocytes from different arterial territories we considered the possibility of a differential content of BK $\beta 1$ transcripts across the arteries under study. To test for this possibility, we measured levels of BK $\beta 1$ mRNA across de-endothelialized ACA, MCA, PCA, BA, MA and CA using quantitative, Real Time PCR. Results demonstrate that BK $\beta 1$ mRNA levels were significantly higher in BA and CA *vs.* those present in all other arteries (Fig. 7). Thus, it is highly likely that the increased BK $\beta 1$ mRNA levels are a primary determinant of the increased BK $\beta 1$ protein levels found in the plasmalemma of BA and CA myocytes (Figs. 5 and 6), an idea that we test below.

Overexpression of BK $\beta 1$ subunit in middle cerebral artery enhances the artery's sensitivity to lithocholate that acts *via* BK $\beta 1$

So far, our collective data indicate that BA and CA are characterized by an increased vasodilatory response to LCA, a $\beta 1$ -dependent BK channel activator, with these arteries being characterized by (a) increased LCA response and (b) basal activity of myocyte BK channels, (c) increased myocyte surface expression of BK $\beta 1$ subunits, and (d) increased levels of BK $\beta 1$ mRNA transcripts. To determine whether increased BK $\beta 1$ mRNA transcript actually leads to increased artery dilation to a BK $\beta 1$ -targeting ligand, we overexpressed BK $\beta 1$ mRNA in rat MCA using *in vitro* cDNA electroporation of the artery (see Material and Methods). MCA was chosen because 1) this artery showed reduced LCA responses (Figs. 1 and 2), BK $\beta 1$ function (Figs. 4, 5 and 6) and BK $\beta 1$ mRNA levels (Fig. 7) when compared to those of BA or CA, 2) rat MCA is a widely used experimental model to address human cerebrovascular pathophysiology and pharmacology [3,26,33,88], and 3) we conducted all our previous studies with smooth muscle BK $\beta 1$ -targeting vasodilators (LCA itself, other

cholanes, LTB₄ and HENA) using rat MCA [15–17,19,63,74]. BK β 1 protein overexpression was confirmed by Western blotting (Fig. 8a). First, arteries that were transfected with BK β 1 cDNA contained significantly higher levels of BK β 1 subunit protein than those of control arteries, i.e., transfected with an “empty” vector (Fig. 8b), underscoring the efficiency of our electroporation procedure. Second, functional studies on pressurized arteries demonstrated that *in vitro* electroporation, either with “empty” vector (pcDNA3 alone) or with β 1 (*KCNMB1*) cDNA, preserved the contractile and dilatory responses evoked by 60 mM K⁺ and Ca²⁺-free bath solution, respectively (Fig. 8c, d). These data underscore that the electroporation procedure did not disrupt maximal contractile/dilatory function of the artery. Third, application of 45 μ M LCA to MCA transfected with “empty” vector resulted in 5.83 $\pm$ 0.4% increase in artery diameter from pre-LCA application level (Fig. 8c, e). This LCA response is similar to that evoked in untransfected MCA (Fig. 8e) and [15]. Finally, and more importantly, MCA transfected with BK β 1 cDNA responded to LCA application with a dilation that reached 10.6 $\pm$ 0.45%, i.e., significantly higher ($p<0.05$) than the dilation of arteries transfected with “empty” vector or the dilation of untransfected arteries (Fig. 8e). Last but not least, the LCA-induced dilation of MCA transfected with BK β 1 cDNA is similar to LCA-induced dilation of arteries that naturally express higher levels of BK β 1 (i.e., BA and CA) (Fig. 1b). These data clearly demonstrate that overexpression of BK β 1 mRNA and, thus, BK β 1 protein levels can improve the vasodilatory response of arteries to BK β 1-dependent vasodilators.

Discussion

Using a combination of quantitative real-time PCR, Western-blotting, laser confocal fluorescence microscopy, single channel resolution patch-clamp electrophysiology, *in vitro* electroporation, and artery pressurization *in vitro*, we demonstrate that arteries from the peripheral (e.g., quaternary branches of MA), coronary, and cerebral (ACA, MCA, PCA, and secondary branches of BA) circulations differ in their dilatory response to a selective BK β 1-targeting vasodilator (LCA). Furthermore, our study reports a differential endothelium-independent vasodilation to LCA across the distribution arteries that stem out of the Willis’ circle, with BA having a response to LCA that is drastically higher than those of ACA, MCA and PCA (Fig. 1B). Biochemical, confocal imaging and electrophysiological data indicate that the arteries with higher responses to LCA, that is CA and BA, also display: (1) enhanced activation of smooth muscle BK channels to this rather selective BK β 1 agonist (Fig. 2 and 3); (2) increased functional presence of BK β 1 subunits in the native smooth muscle BK channel (Fig. 4); (3) increased immunofluorescence for BK β 1 subunit protein in the myocyte plasmalemma-subplasmalemma region (Figs. 5 and 6), and (4) significantly higher levels of BK β 1 mRNA in cerebral smooth muscle (Fig. 7). All these functional and biochemical differences reflecting a differential BK β 1 subunit functional presence across different vessels seems rather selective, as they are not accompanied by detectable changes in expression, membrane location or mRNA levels of the BK channel-forming α subunit. Conceivably, the increase in mRNA BK β 1 transcript in vascular smooth muscle is the major mechanism leading to enhanced smooth muscle BK channel function and eventual increased dilation of BA and CA in response to a selective BK β 1-targeting physiological agent. Indeed, our study proves that transfecting BK- β 1 coding cDNA into MCA, an artery that has

much lower levels of BK $\beta 1$ and LCA-invoked dilation when compared to CA or BA, increases the dilatory of MCA to levels similar to those of CA and BA (Fig. 8).

A previous study using rats has documented a higher functional expression of BK $\beta 1$ in cerebral artery (territory unspecified) arterioles vs. primary and secondary branches of cremaster artery [91]. Likewise, smooth muscle BK channels in C57/BL6 mouse cerebral arteries show a higher functional presence of BK $\beta 1$ when compared to pulmonary artery myocytes [94]. Notably, these two studies evaluated smooth muscle BK channels in arterial segments that greatly differ in diameter and/or function: mouse cerebral arteries have a diameter of $\sim 100 \mu\text{m}$ and serve as resistance vessels whereas their pulmonary counterparts have a diameter of $\sim 800 \mu\text{m}$ and serve as conduit vessels [94]. Rat MCA main branches and first order cremaster arterioles also greatly differ in diameter: ~ 284 and $168 \mu\text{m}$ (passive diameter), respectively [42]. Our data, however, document that differences in functional BK $\beta 1$ presence in smooth muscle from different territories can be observed across arteries that: (a) were specifically selected to fall within a similar diameter range ($175\text{--}230 \mu\text{m}$). Indeed, secondary and quaternary branches of BA and MA respectively, were dissected whereas primary branches of ACA, MCA, PCA were chosen (i.e., ACA: $210.97 \pm 13.69 \mu\text{m}$; MCA: $174.73 \pm 11.29 \mu\text{m}$; PCA: $193.08 \pm 17.08 \mu\text{m}$; BA: $179.08 \pm 23.79 \mu\text{m}$; MA: $229.83 \pm 25.04 \mu\text{m}$; CA: $223.22 \pm 29.52 \mu\text{m}$); (b) are exposed to similar levels and variations in systemic arterial pressure, i.e., CA vs. MA quaternary branches, or (c) fulfill an equivalent function, i.e., primary blood distribution branching out of the Willis' circle, i.e., BA vs. ACA, MCA and PCA. Remarkably, our study reveals that the arteries with higher functional BK $\beta 1$ expression irrigate vital organs: CA provides blood to the myocardium while BA, together with vertebral arteries, the brainstem [24,38,53], which includes cardio-respiratory centers essential for life. Since BK $\beta 1$ subunits are not found in invertebrates, such as *D. melanogaster* or *C. elegans* [23,71,95], it is tempting to speculate that the high functional expression of BK $\beta 1$ subunit in rat CA and BA smooth muscle could be indicative of an enhanced protection against vasoconstriction in organs and brain regions that are vital to more evolutionary-advanced organisms, including mammals.

The increased functional presence of BK $\beta 1$ subunits in the smooth muscle of rat CA and BA when compared to other arterial territories is evident from the leftward shift in the $\text{NP}_0/\text{NP}_0\text{-V}$ plots with the resulting decrease in V_{half} (Fig. 4a, b), and the increased response to $45 \mu\text{M}$ LCA (Figs. 2 and 3), that is, a concentration that requires the presence of functional $\beta 1$ subunits in the smooth muscle BK channel complex for LCA to increase channel activity and thus evoke endothelium-independent MCA dilation in both rat and mouse [15]. A similar combination of patch-clamp electrophysiology and pharmacological profiling (i.e., application of 17β -estradiol) was previously used to conclude that cerebral artery BK channels contain functional levels of $\beta 1$ subunits higher than those found in the cremaster arteries. Consistently, Western-blotting results have shown higher levels of BK $\beta 1$ protein in the cerebral arteries [91]. Our laser confocal microscopy data further document that, when compared to myocytes from other arteries, the increased functional presence of BK $\beta 1$ subunits in myocytes from BA and CA is associated with increased levels of this subunit in the myocyte *plasmalemma-subplasmalemma*, in absence of detectable changes in α subunit location or expression.

Several non-mutually exclusive mechanisms may account for the increased presence of BK $\beta 1$ subunits in myocytes of CA and BA arteries, including increased subunit shuttling from the endoplasmic reticulum-Golgi to the plasmalemma, decreased endocytosis and degradation, and increased gene expression. While changes in BK $\beta 1$ subunit between the cell membrane and internal compartments [55] cannot be rule out, the fact that CA and BA present drastically higher levels of cDNA coding for BK $\beta 1$ (Fig. 7) supports the idea that the increased BK $\beta 1$ protein in the membrane of CA or BA myocytes is driven by increased levels in BK $\beta 1$ mRNA transcripts. Consistent with this hypothesis, the LCA-induced dilation of MCA (which shows lower cDNA $\beta 1$ levels than those of BA or CA; Fig. 7) transfected with BK $\beta 1$ cDNA is similar to the dilation of arteries that naturally express higher levels of BK $\beta 1$ (i.e., BA and CA) (Fig. 8d, e).

It was initially thought that the stoichiometry of assembly of α and $\beta 1$ subunits is 1:1 [47], resulting in a hetero-octameric protein complex (i.e., $4\alpha:4\beta 1$). Using recombinant α and $\beta 1$ subunits expressed in *Xenopus laevis* oocytes, however, it has been demonstrated that functional channels may include a variant number of $\beta 1$ subunits. Moreover, each individual $\beta 1$ subunit inserted in the channel complex produces an essentially identical, incremental effect on channel gating and thus, availability for activation [89]. These authors reported that four different $\alpha:\beta 1$ molar ratios (reflecting stoichiometries between $4\alpha:1\beta 1$ and $4\alpha:4\beta 1$) rendered four distinct current phenotypes independently of the α subunit used. Conceivably, the reduced activity and LCA responses of myocyte BK channels from ACA, MCA, PCA and MA may be determined by channel complexes that are unsaturated with $\beta 1$ proteins, with the $4\alpha:4\beta 1$ stoichiometry being predominant in myocyte BK channels from BA, CA and from MCA transfected with $\beta 1$ BK cDNA. Disregarding the stoichiometric underpinnings that may contribute to the increased activity and LCA responses of myocyte BK channels from CA and BA, our results clearly indicate that basal activity and the pharmacological response of vascular smooth muscle BK channels can be substantially regulated by manipulating the expression of the BK $\beta 1$ subunits.

While presenting some regional variations, the major cerebral arteries of rats and humans share strong similarities in overall organization, vessel structure, and morphological changes driven by cerebrovascular disease [36,53]. Thus, our current findings may have wide pathophysiological and health-related implications, as follows. Consistent with previous reports [15,16], our study shows that $45\text{ }\mu\text{M}$ LCA evokes an increase in MCA diameter of $4.59\pm 0.85\%$. In contrast, this LCA concentration resulted in a $12.98\pm 1.5\%$ increase in BA diameter (Fig. 1b). Poiseuille's law has established that fluid flow is a function of the 4th power of the vessel radius. Thus, LCA-induced vasodilation should result in a robust increase in cerebral blood flow: about +19.7% and +63% for territories irrigated by MCA and BA, respectively. Under physiological conditions, LCA in particular and bile acids in general barely reach 2–3 μM in systemic (including cerebral) circulation [21,39,40,77]. However, many human pathological conditions including liver cirrhosis [45,46,58], chronic active hepatitis [49], extrahepatic cholestasis [58] and the stagnant-loop syndrome [56] are associated with abnormal levels of bile acids in systemic blood due to spillover of bile acids from the portal to the systemic circulation [37,41,56,82]. Such spillover results in a 10–100-fold higher systemic bile acid concentration [21,37,43,72]. These abnormal levels of bile acids are responsible for the systemic hypotension observed in patients with liver damage

and/or significant portosystemic circulatory shunt [8]. Moreover, several experimental models have demonstrated that bile acids evoke vasodilation in an endothelium-independent manner, attenuate vascular reactivity *in vitro*, and reduce systemic blood pressure *in vivo* [9,15,73]. Our previous studies have shown that dilation of rat and mouse MCA by bile acids, LCA in particular, is mediated by a direct interaction between LCA and the BK channel $\beta 1$ protein [15,17,19,20]. Our current data suggest that territories irrigated by the vertebro-basilar system, such as the medulla, cerebellum, pons, midbrain, thalamus, and occipital cortex, which control heart rate, respiration, gait and equilibrium, audition, memory and sleep cycling would be the most affected by arterial dilation in response to μM levels of circulating bile acids. Bile acids have been increasingly recognized as major pathophysiological players in neurological disease (reviewed in [64]). An arterial contribution to such bile acid role, however, remains to be studied.

In turn, LCA synthetic analogs that selectively target BK smooth muscle $\beta 1$ subunits have been developed in the hope of obtaining cerebral vasodilators that remain effective independently of intact endothelial function, and lack side-effects related to steroidal nucleus recognition [16]. The need for such development is compounded by the facts that (a) biomedical research has largely failed to produce effective, easy to use, cerebral vasodilators [54], and (b) endothelial-mediated vasodilation is impaired in various conditions associated with cerebrovascular ischemia, such as atherosclerosis. Our current data suggest that such BK $\beta 1$ -targeting smooth muscle relaxants (e.g., HENA; [16]) would be more effective in evoking dilation of the vertebro-basilar system than of ACA, PCA or MCA. The use of HENA or other LCA-related agents that evoke endothelium-independent dilation, however, should be hampered under conditions where the functional presence of BK $\beta 1$ subunits is diminished. For instance, down-regulation of these subunits has been reported in rats subject to angiotensin-induced hypertension [2], streptozocin-induced diabetic hypertension [62,92,93], and even with ageing [61,69,83]. Interestingly, it has been reported that $\text{NO}\bullet$ and other smooth muscle relaxants dilate cerebral arteries by altering the shuttling of BK $\beta 1$ subunits with eventual increased location of these subunits in the myocyte plasmalemma [55]. Then, $\text{NO}\bullet$ -delivering agents and HENA-like agents might constitute an attractive combination to evoke cerebral dilation under the aforementioned conditions associated with reduced amount of BK $\beta 1$ subunits.

Our present results also suggest that in peripheral circulation, BK $\beta 1$ -targeting vasodilators, whether endogenous or exogenous, would be more effective on CA than on MA arteries. Interestingly, 17β estradiol has been reported to increase BK channel function and, thus, relax vascular smooth muscle, *via* several mechanisms, including a direct interaction with BK $\beta 1$ subunits [84,86]. Thus, it is possible to speculate that CA relaxation as a result of 17β estradiol-BK $\beta 1$ direct interaction could be particularly effective to protect the myocardium against ischemia.

Finally, previous studies found that a polymorphism (E65K) in KCNMB1 resulted in a gain-of-function BK channel, such polymorphism being associated with protection against moderate-to-severe diastolic hypertension. The E65K substitution in BK $\beta 1$ subunits resulted in heteromeric channels that show an increased Ca^{2+} sensitivity, which would result in a more efficient negative feedback on vascular smooth muscle contractility [32].

Considering the highest level of $\beta 1$ subunits in CA compared to most other vascular beds (Figs. 5 and 6), it can be hypothesized that CA would be particularly susceptible to such polymorphism. Interestingly, E65K carriers in the older female population show a drastically reduced occurrence of myocardial infarction when compared to non-carriers [78].

In synthesis, we have demonstrated that BK $\beta 1$ subunits show marked differences in both gene and protein expression across intracranial (ACA, MCA and PCA vs. BA) and peripheral (CA vs. MA) vessels, with CA and BA showing the highest levels. Further, these differences in transcript and protein levels translate into higher basal BK channel activity as well as increased channel activation and vasodilatory responses to an endogenous BK $\beta 1$ -selective agonist. Thus, our study buttresses the notion that BK $\beta 1$ subunits exhibit a different functional presence across arterial territories, which can explain differential local responses to endogenous and exogenous vasodilators, and may provide a molecular basis for regional susceptibility to vascular disease.

Acknowledgments

Authors thank Bangalore Shivakumar and Maria T. Asuncion-Chin for their excellent technical assistance. This work was supported by an American Heart Association Predoctoral Fellowship (to G.K.), and National Institutes of Health grants R37AA11560 and R01HL104631 (to A.M.D). Authors declare no competing financial interests.

References

1. Amberg GC, Bonev AD, Rossow CF, Nelson MT, Santana LF. Modulation of the molecular composition of large conductance, $\text{Ca}(2+)$ activated $\text{K}(+)$ channels in vascular smooth muscle during hypertension. *The Journal of clinical investigation*. 2003; 112:717–724. DOI: 10.1172/jci18684 [PubMed: 12952920]
2. Amberg GC, Santana LF. Downregulation of the BK channel beta1 subunit in genetic hypertension. *Circulation research*. 2003; 93:965–971. DOI: 10.1161/01.RES.0000100068.43006.36 [PubMed: 14551242]
3. Arab L, Liebeskind DS. Tea, flavonoids and stroke in man and mouse. *Archives of biochemistry and biophysics*. 2010; 501:31–36. DOI: 10.1016/j.abb.2010.03.015 [PubMed: 20361928]
4. Bannister JP, Adebisi A, Zhao G, Narayanan D, Thomas CM, Feng JY, Jaggar JH. Smooth muscle cell $\alpha 2\delta$ -1 subunits are essential for vasoregulation by $\text{CaV}1.2$ channels. *Circulation research*. 2009; 105:948–955. DOI: 10.1161/circresaha.109.203620 [PubMed: 19797702]
5. Bao L, Cox DH. Gating and ionic currents reveal how the BKCa channel's Ca^{2+} sensitivity is enhanced by its beta1 subunit. *The Journal of general physiology*. 2005; 126:393–412. DOI: 10.1085/jgp.200509346 [PubMed: 16186565]
6. Barkoudah E, Jaggar JH, Leffler CW. The permissive role of endothelial NO in CO-induced cerebrovascular dilation. *American journal of physiology Heart and circulatory physiology*. 2004; 287:H1459–1465. DOI: 10.1152/ajpheart.00369.2004 [PubMed: 15191891]
7. Behrens R, Nolting A, Reimann F, Schwarz M, Waldschutz R, Pongs O. hKCNMB3 and hKCNMB4, cloning and characterization of two members of the large-conductance calcium-activated potassium channel beta subunit family. *FEBS letters*. 2000; 474:99–106. [PubMed: 10828459]
8. Bomzon A, Finberg JP, Tovbin D, Naidu SG, Better OS. Bile salts, hypotension and obstructive jaundice. *Clinical science (London, England : 1979)*. 1984; 67:177–183.
9. Bomzon A, Ljubuncic P. Bile acids as endogenous vasodilators? *Biochemical pharmacology*. 1995; 49:581–589. [PubMed: 7887972]
10. Brayden JE, Nelson MT. Regulation of arterial tone by activation of calcium-dependent potassium channels. *Science*. 1992; 256:532–535. [PubMed: 1373909]

11. Brenner R, Jegla TJ, Wickenden A, Liu Y, Aldrich RW. Cloning and functional characterization of novel large conductance calcium-activated potassium channel beta subunits, hKCNMB3 and hKCNMB4. *The Journal of biological chemistry*. 2000; 275:6453–6461. [PubMed: 10692449]
12. Brenner R, Perez GJ, Bonev AD, Eckman DM, Kosek JC, Wiler SW, Patterson AJ, Nelson MT, Aldrich RW. Vasoregulation by the beta1 subunit of the calcium-activated potassium channel. *Nature*. 2000; 407:870–876. DOI: 10.1038/35038011 [PubMed: 11057658]
13. Bukiya A, Dopico AM, Leffler CW, Fedinec A. Dietary cholesterol protects against alcohol-induced cerebral artery constriction. *Alcoholism, clinical and experimental research*. 2014; 38:1216–1226. DOI: 10.1111/acer.12373
14. Bukiya AN, Liu J, Dopico AM. The BK channel accessory beta1 subunit determines alcohol-induced cerebrovascular constriction. *FEBS letters*. 2009; 583:2779–2784. DOI: 10.1016/j.febslet.2009.07.019 [PubMed: 19616547]
15. Bukiya AN, Liu J, Toro L, Dopico AM. Beta1 (KCNMB1) subunits mediate lithocholate activation of large-conductance Ca²⁺-activated K⁺ channels and dilation in small, resistance-size arteries. *Molecular pharmacology*. 2007; 72:359–369. DOI: 10.1124/mol.107.034330 [PubMed: 17468198]
16. Bukiya AN, McMillan JE, Fedinec AL, Patil SA, Miller DD, Leffler CW, Parrill AL, Dopico AM. Cerebrovascular dilation via selective targeting of the cholane steroid-recognition site in the BK channel beta1-subunit by a novel nonsteroidal agent. *Molecular pharmacology*. 2013; 83:1030–1044. DOI: 10.1124/mol.112.083519 [PubMed: 23455312]
17. Bukiya AN, Singh AK, Parrill AL, Dopico AM. The steroid interaction site in transmembrane domain 2 of the large conductance, voltage- and calcium-gated potassium (BK) channel accessory beta1 subunit. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108:20207–20212. DOI: 10.1073/pnas.1112901108 [PubMed: 22123969]
18. Bukiya AN, Vaithianathan T, Kuntamallappanavar G, Asuncion-Chin M, Dopico AM. Smooth muscle cholesterol enables BK beta1 subunit-mediated channel inhibition and subsequent vasoconstriction evoked by alcohol. *Arteriosclerosis, thrombosis, and vascular biology*. 2011; 31:2410–2423. DOI: 10.1161/atvbaha.111.233965
19. Bukiya AN, Vaithianathan T, Toro L, Dopico AM. The second transmembrane domain of the large conductance, voltage- and calcium-gated potassium channel beta(1) subunit is a lithocholate sensor. *FEBS letters*. 2008; 582:673–678. DOI: 10.1016/j.febslet.2008.01.036 [PubMed: 18242174]
20. Bukiya AN, Vaithianathan T, Toro L, Dopico AM. Channel beta2–4 subunits fail to substitute for beta1 in sensitizing BK channels to lithocholate. *Biochemical and biophysical research communications*. 2009; 390:995–1000. DOI: 10.1016/j.bbrc.2009.10.091 [PubMed: 19852931]
21. Ceryak S, Bouscarel B, Fromm H. Comparative binding of bile acids to serum lipoproteins and albumin. *J Lipid Res*. 1993; 34:1661–1674. [PubMed: 8245717]
22. Chang J, Fedinec AL, Kuntamallappanavar G, Leffler CW, Bukiya AN, Dopico AM. Endothelial Nitric Oxide Mediates Caffeine Antagonism of Alcohol-Induced Cerebral Artery Constriction. *The Journal of pharmacology and experimental therapeutics*. 2016; 356:106–115. DOI: 10.1124/jpet.115.229054 [PubMed: 26555891]
23. Chen B, Ge Q, Xia XM, Liu P, Wang SJ, Zhan H, Eipper BA, Wang ZW. A novel auxiliary subunit critical to BK channel function in *Caenorhabditis elegans*. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2010; 30:16651–16661. DOI: 10.1523/jneurosci.3211-10.2010 [PubMed: 21148004]
24. Cho L, Mukherjee D. Basic cerebral anatomy for the carotid interventionalist: the intracranial and extracranial vessels. *Catheterization and cardiovascular interventions : official journal of the Society for Cardiac Angiography & Interventions*. 2006; 68:104–111. DOI: 10.1002/ccd.20712 [PubMed: 16763991]
25. Chomczynski P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *BioTechniques*. 1993; 15:532–534. 536–537. [PubMed: 7692896]
26. Cipolla MJ, Curry AB. Middle cerebral artery function after stroke: the threshold duration of reperfusion for myogenic activity. *Stroke; a journal of cerebral circulation*. 2002; 33:2094–2099.

27. Cox DH, Aldrich RW. Role of the beta1 subunit in large-conductance Ca(2+)-activated K(+) channel gating energetics. Mechanisms of enhanced Ca(2+) sensitivity. *The Journal of general physiology*. 2000; 116:411–432. [PubMed: 10962017]
28. Dopico AM. Ethanol sensitivity of BK(Ca) channels from arterial smooth muscle does not require the presence of the beta 1-subunit. *American journal of physiology Cell physiology*. 2003; 284:C1468–1480. DOI: 10.1152/ajpcell.00421.2002 [PubMed: 12570985]
29. Dopico AM, Anantharam V, Treistman SN. Ethanol increases the activity of Ca(++)-dependent K+ (mslo) channels: functional interaction with cytosolic Ca++ *The Journal of pharmacology and experimental therapeutics*. 1998; 284:258–268. [PubMed: 9435186]
30. Dopico AM, Bukiya AN. Lipid regulation of BK channel function. *Frontiers in physiology*. 2014; 5:312.doi: 10.3389/fphys.2014.00312 [PubMed: 25202277]
31. Dopico AM, Kirber MT, Singer JJ, Walsh JV Jr. Membrane stretch directly activates large conductance Ca(2+)-activated K+ channels in mesenteric artery smooth muscle cells. *American journal of hypertension*. 1994; 7:82–89. [PubMed: 8136116]
32. Fernandez-Fernandez JM, Tomas M, Vazquez E, Orio P, Latorre R, Senti M, Marrugat J, Valverde MA. Gain-of-function mutation in the KCNMB1 potassium channel subunit is associated with low prevalence of diastolic hypertension. *The Journal of clinical investigation*. 2004; 113:1032–1039. DOI: 10.1172/jci20347 [PubMed: 15057310]
33. Fluri F, Schuhmann MK, Kleinschnitz C. Animal models of ischemic stroke and their application in clinical research. *Drug design, development and therapy*. 2015; 9:3445–3454. DOI: 10.2147/dddt.s56071
34. Geary GG, Osol GJ, Longo LD. Development affects in vitro vascular tone and calcium sensitivity in ovine cerebral arteries. *The Journal of physiology*. 2004; 558:883–896. DOI: 10.1113/jphysiol.2003.056945 [PubMed: 15131239]
35. Gollasch M, Wellman GC, Knot HJ, Jaggar JH, Damon DH, Bonev AD, Nelson MT. Ontogeny of local sarcoplasmic reticulum Ca2+ signals in cerebral arteries: Ca2+ sparks as elementary physiological events. *Circulation research*. 1998; 83:1104–1114. [PubMed: 9831705]
36. Gray, H., Bannister, LH., Berry, MM., Williams, PL. *Gray's Anatomy, the Anatomical basis of surgery and Medicine*. 38. Elsevier Science; New York: 1995.
37. Greco AV, Mingrone G. Serum bile acid concentrations in mild liver cirrhosis. *Clinica chimica acta; international journal of clinical chemistry*. 1993; 221:183–189. [PubMed: 8149635]
38. Greene, EC. *Anatomy of the Rat*. Hafner; 1963.
39. Guldutuna S, Leuschner M, Wunderlich N, Nickel A, Bhatti S, Hubner K, Leuschner U. Cholic acid and ursodeoxycholic acid therapy in primary biliary cirrhosis. Changes in bile acid patterns and their correlation with liver function. *European journal of clinical pharmacology*. 1993; 45:221–225. [PubMed: 8276045]
40. Guldutuna S, You T, Kurts W, Leuschner U. High performance liquid chromatographic determination of free and conjugated bile acids in serum, liver biopsies, bile, gastric juice and feces by fluorescence labeling. *Clinica chimica acta; international journal of clinical chemistry*. 1993; 214:195–207. [PubMed: 8472385]
41. Hamdan H, Stacey NH. Mechanism of trichloroethylene-induced elevation of individual serum bile acids. I. Correlation of trichloroethylene concentrations to bile acids in rat serum. *Toxicology and applied pharmacology*. 1993; 121:291–295. DOI: 10.1006/taap.1993.1156 [PubMed: 8346545]
42. Howitt L, Sandow SL, Grayson TH, Ellis ZE, Morris MJ, Murphy TV. Differential effects of diet-induced obesity on BKCa {beta} 1-subunit expression and function in rat skeletal muscle arterioles and small cerebral arteries. *American journal of physiology Heart and circulatory physiology*. 2011; 301:H29–40. DOI: 10.1152/ajpheart.00134.2011 [PubMed: 21536854]
43. JO. *Hepatic Transport and Bile Secretion: Physiology and Pathophysiology*. Raven Press; New York: 1993. Metabolism of bile salts in cholestasis in humans.
44. Jaggar JH, Porter VA, Lederer WJ, Nelson MT. Calcium sparks in smooth muscle. *American journal of physiology Cell physiology*. 2000; 278:C235–256. [PubMed: 10666018]
45. Jonsson G, Hedenborg G, Wisen O, Norman A. Presence of bile acid metabolites in serum, urine, and faeces in cirrhosis. *Scandinavian journal of clinical and laboratory investigation*. 1992; 52:555–564. [PubMed: 1411266]

46. Jonsson G, Hedenborg G, Wisen O, Norman A. Serum concentrations and excretion of bile acids in cirrhosis. *Scandinavian journal of clinical and laboratory investigation*. 1992; 52:599–605. [PubMed: 1455151]
47. Knaus HG, Folander K, Garcia-Calvo M, Garcia ML, Kaczorowski GJ, Smith M, Swanson R. Primary sequence and immunological characterization of beta-subunit of high conductance $\text{Ca}(2+)\text{-activated K}^+$ channel from smooth muscle. *The Journal of biological chemistry*. 1994; 269:17274–17278. [PubMed: 8006036]
48. Knot HJ, Nelson MT. Regulation of arterial diameter and wall $[\text{Ca}^{2+}]$ in cerebral arteries of rat by membrane potential and intravascular pressure. *The Journal of physiology*. 1998; 508(Pt 1):199–209. [PubMed: 9490839]
49. Korman MG, Hofmann AF, Summerskill WH. Assessment of activity in chronic active liver disease. Serum bile acids compared with conventional tests and histology. *The New England journal of medicine*. 1974; 290:1399–1402. DOI: 10.1056/nejm197406202902503 [PubMed: 4133590]
50. Kuntamallappanavar G, Dopico AM. Alcohol modulation of BK channel gating depends on beta subunit composition. *The Journal of general physiology*. 2016; 148:419–440. DOI: 10.1085/jgp.201611594 [PubMed: 27799321]
51. Kuntamallappanavar G, Toro L, Dopico AM. Both Transmembrane Domains of BK beta1 Subunits Are Essential to Confer the Normal Phenotype of beta1-Containing BK Channels. *PloS one*. 2014; 9:e109306.doi: 10.1371/journal.pone.0109306 [PubMed: 25275635]
52. Ledoux J, Werner ME, Brayden JE, Nelson MT. Calcium-activated potassium channels and the regulation of vascular tone. *Physiology*. 2006; 21:69–78. DOI: 10.1152/physiol.00040.2005 [PubMed: 16443824]
53. Lee RM. Morphology of cerebral arteries. *Pharmacology & therapeutics*. 1995; 66:149–173. [PubMed: 7630927]
54. Legos JJ, Tuma RF, Barone FC. Pharmacological interventions for stroke: failures and future. *Expert opinion on investigational drugs*. 2002; 11:603–614. DOI: 10.1517/13543784.11.5.603 [PubMed: 11996643]
55. Leo MD, Bannister JP, Narayanan D, Nair A, Grubbs JE, Gabrick KS, Boop FA, Jaggar JH. Dynamic regulation of beta1 subunit trafficking controls vascular contractility. *Proceedings of the National Academy of Sciences of the United States of America*. 2014; 111:2361–2366. DOI: 10.1073/pnas.1317527111 [PubMed: 24464482]
56. Lewis B, Tabaqchali S, Panveliwalla D, Wootton ID. Serum-bile-acids in the stagnant-loop syndrome. *Lancet*. 1969; 1:219–220. [PubMed: 4178587]
57. Li B, Jie W, Huang L, Wei P, Li S, Luo Z, Friedman AK, Meredith AL, Han MH, Zhu XH, Gao TM. Nuclear BK channels regulate gene expression via the control of nuclear calcium signaling. *Nature neuroscience*. 2014; 17:1055–1063. DOI: 10.1038/nn.3744 [PubMed: 24952642]
58. Lindblad L, Lundholm K, Schersten T. Bile acid concentrations in systemic and portal serum in presumably normal man and in cholestatic and cirrhotic conditions. *Scandinavian journal of gastroenterology*. 1977; 12:395–400. [PubMed: 560715]
59. Liu P, Xi Q, Ahmed A, Jaggar JH, Dopico AM. Essential role for smooth muscle BK channels in alcohol-induced cerebrovascular constriction. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101:18217–18222. DOI: 10.1073/pnas.0406096102 [PubMed: 15604147]
60. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-(\Delta\Delta C_T)}$ Method. *Methods (San Diego, Calif)*. 2001; 25:402–408. DOI: 10.1006/meth.2001.1262
61. Marijic J, Li Q, Song M, Nishimaru K, Stefani E, Toro L. Decreased expression of voltage- and $\text{Ca}(2+)\text{-activated K}^+$ channels in coronary smooth muscle during aging. *Circulation research*. 2001; 88:210–216. [PubMed: 11157674]
62. McGahon MK, Zhang X, Scholfield CN, Curtis TM, McGeown JG. Selective downregulation of the BKbeta1 subunit in diabetic arteriolar myocytes. *Channels (Austin)*. 2007; 1:141–143. [PubMed: 18690028]

63. McMillan JE, Bukiya AN, Terrell CL, Patil SA, Miller DD, Dopico AM, Parrill AL. Multi-generational pharmacophore modeling for ligands to the cholane steroid-recognition site in the beta(1) modulatory subunit of the BKCa channel. *Journal of molecular graphics & modelling*. 2014; 54:174–183. DOI: 10.1016/j.jmgm.2014.10.008 [PubMed: 25459769]
64. McMillin M, DeMorrow S. Effects of bile acids on neurological function and disease. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2016; 30:3658–3668. DOI: 10.1096/fj.201600275R [PubMed: 27468758]
65. Meera P, Wallner M, Jiang Z, Toro L. A calcium switch for the functional coupling between alpha (hslo) and beta subunits (KV,Ca beta) of maxi K channels. *FEBS letters*. 1996; 382:84–88. [PubMed: 8612769]
66. Meera P, Wallner M, Toro L. A neuronal beta subunit (KCNMB4) makes the large conductance, voltage- and Ca²⁺-activated K⁺ channel resistant to charybdotoxin and iberiotoxin. *Proceedings of the National Academy of Sciences of the United States of America*. 2000; 97:5562–5567. DOI: 10.1073/pnas.100118597 [PubMed: 10792058]
67. Nimigean CM, Magleby KL. The beta subunit increases the Ca²⁺ sensitivity of large conductance Ca²⁺-activated potassium channels by retaining the gating in the bursting states. *The Journal of general physiology*. 1999; 113:425–440. [PubMed: 10051518]
68. Nimigean CM, Magleby KL. Functional coupling of the beta(1) subunit to the large conductance Ca(2+)-activated K(+) channel in the absence of Ca(2+). Increased Ca(2+) sensitivity from a Ca(2+)-independent mechanism. *The Journal of general physiology*. 2000; 115:719–736. [PubMed: 10828246]
69. Nishimaru K, Eghbali M, Lu R, Marijic J, Stefani E, Toro L. Functional and molecular evidence of MaxiK channel beta1 subunit decrease with coronary artery ageing in the rat. *The Journal of physiology*. 2004; 559:849–862. DOI: 10.1113/jphysiol.2004.068676 [PubMed: 15272033]
70. Orio P, Latorre R. Differential effects of beta 1 and beta 2 subunits on BK channel activity. *The Journal of general physiology*. 2005; 125:395–411. DOI: 10.1085/jgp.200409236 [PubMed: 15767297]
71. Orio P, Rojas P, Ferreira G, Latorre R. New disguises for an old channel: MaxiK channel beta-subunits. *News in physiological sciences : an international journal of physiology produced jointly by the International Union of Physiological Sciences and the American Physiological Society*. 2002; 17:156–161.
72. Ostrow, J. *Hepatic Transport and Bile Secretion: Physiology and Pathophysiology*. Raven; New York: 1993. Metabolism of bile salts in cholestasis in humans.
73. Pak JM, Lee SS. Vasoactive effects of bile salts in cirrhotic rats: in vivo and in vitro studies. *Hepatology (Baltimore, Md)*. 1993; 18:1175–1181.
74. Patil S, Bukiya AN, Li W, Dopico AM, Miller D. Design and synthesis of hydroxy-alkynoic acids and their methyl esters as novel activators of BK channels. *Bioorganic & medicinal chemistry letters*. 2008; 18:3427–3430. DOI: 10.1016/j.bmcl.2008.03.080 [PubMed: 18462939]
75. Patterson AJ, Henrie-Olson J, Brenner R. Vasoregulation at the molecular level: a role for the beta1 subunit of the calcium-activated potassium (BK) channel. *Trends in cardiovascular medicine*. 2002; 12:78–82. [PubMed: 11852255]
76. Perez GJ, Bonev AD, Nelson MT. Micromolar Ca(2+) from sparks activates Ca(2+)-sensitive K(+) channels in rat cerebral artery smooth muscle. *American journal of physiology Cell physiology*. 2001; 281:C1769–1775. [PubMed: 11698234]
77. Scharschmidt BF, Blanckaert N, Farina FA, Kabra PM, Stafford BE, Weisiger RA. Measurement of serum bilirubin and its mono- and diconjugates: application to patients with hepatobiliary disease. *Gut*. 1982; 23:643–649. [PubMed: 7095558]
78. Senti M, Fernandez-Fernandez JM, Tomas M, Vazquez E, Elosua R, Marrugat J, Valverde MA. Protective effect of the KCNMB1 E65K genetic polymorphism against diastolic hypertension in aging women and its relevance to cardiovascular risk. *Circulation research*. 2005; 97:1360–1365. DOI: 10.1161/01.res.0000196557.93717.95 [PubMed: 16293791]
79. Siemen D, Loupatatzis C, Borecky J, Gulbins E, Lang F. Ca²⁺-activated K channel of the BK-type in the inner mitochondrial membrane of a human glioma cell line. *Biochemical and biophysical research communications*. 1999; 257:549–554. [PubMed: 10198249]

80. Singh H, Lu R, Bopassa JC, Meredith AL, Stefani E, Toro L. MitoBK(Ca) is encoded by the *Kcnma1* gene, and a splicing sequence defines its mitochondrial location. *Proceedings of the National Academy of Sciences of the United States of America*. 2013; 110:10836–10841. DOI: 10.1073/pnas.1302028110 [PubMed: 23754429]
81. Soni H, Buddington RK, Adebisi A. Postnatal kidney maturation regulates renal artery myogenic constriction. *Journal of perinatal medicine*. 2015; 43:119–122. DOI: 10.1515/jpm-2013-0346 [PubMed: 24897391]
82. Tarantino G, Cambri S, Ferrara A, Marzano M, Liberti A, Vellone G, Ciccarelli AFL. Concentrazione sierica degli acidi biliari e ipertensione portale di pazienti cirrotici. Possibile correlazione. *Eur Rev Med Pharmacol Sci*. 1989; 11:195–205.
83. Toro L, Marijic J, Nishimaru K, Tanaka Y, Song M, Stefani E. Aging, ion channel expression, and vascular function. *Vascular pharmacology*. 2002; 38:73–80.
84. Torres YP, Granados ST, Latorre R. Pharmacological consequences of the coexpression of BK channel alpha and auxiliary beta subunits. *Frontiers in physiology*. 2014; 5:383.doi: 10.3389/fphys.2014.00383 [PubMed: 25346693]
85. Uebele VN, Lagrutta A, Wade T, Figueroa DJ, Liu Y, McKenna E, Austin CP, Bennett PB, Swanson R. Cloning and functional expression of two families of beta-subunits of the large conductance calcium-activated K⁺ channel. *The Journal of biological chemistry*. 2000; 275:23211–23218. DOI: 10.1074/jbc.M910187199 [PubMed: 10766764]
86. Valverde MA, Rojas P, Amigo J, Cosmelli D, Orio P, Bahamonde MI, Mann GE, Vergara C, Latorre R. Acute activation of Maxi-K channels (hSlo) by estradiol binding to the beta subunit. *Science*. 1999; 285:1929–1931. [PubMed: 10489376]
87. Wallner M, Meera P, Toro L. Molecular basis of fast inactivation in voltage and Ca²⁺-activated K⁺ channels: a transmembrane beta-subunit homolog. *Proceedings of the National Academy of Sciences of the United States of America*. 1999; 96:4137–4142. [PubMed: 10097176]
88. Wang-Fischer YKL. Animal models of ischemic stroke: a historical survey. *Manual of Stroke Models in Rats*. 2009:5–11.
89. Wang YW, Ding JP, Xia XM, Lingle CJ. Consequences of the stoichiometry of Slo1 alpha and auxiliary beta subunits on functional properties of large-conductance Ca²⁺-activated K⁺ channels. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2002; 22:1550–1561. [PubMed: 11880485]
90. Xia XM, Ding JP, Lingle CJ. Molecular basis for the inactivation of Ca²⁺- and voltage-dependent BK channels in adrenal chromaffin cells and rat insulinoma tumor cells. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 1999; 19:5255–5264. [PubMed: 10377337]
91. Yang Y, Sohma Y, Nourian Z, Ella SR, Li M, Stupica A, Korthuis RJ, Davis MJ, Braun AP, Hill MA. Mechanisms underlying regional differences in the Ca²⁺ sensitivity of BK(Ca) current in arteriolar smooth muscle. *The Journal of physiology*. 2013; 591:1277–1293. DOI: 10.1113/jphysiol.2012.241562 [PubMed: 23297302]
92. Ye CL, Shen B, Ren XD, Luo RJ, Ding SY, Yan FM, Jiang JH. An increase in opening of BK(Ca) channels in smooth muscle cells in streptozotocin-induced diabetic mice. *Acta pharmacologica Sinica*. 2004; 25:744–750. [PubMed: 15169626]
93. Yi F, Wang H, Chai Q, Wang X, Shen WK, Willis MS, Lee HC, Lu T. Regulation of large conductance Ca²⁺-activated K⁺ (BK) channel beta1 subunit expression by muscle RING finger protein 1 in diabetic vessels. *The Journal of biological chemistry*. 2014; 289:10853–10864. DOI: 10.1074/jbc.M113.520940 [PubMed: 24570002]
94. Zheng YM, Park SW, Stokes L, Tang Q, Xiao JH, Wang YX. Distinct activity of BK channel beta1-subunit in cerebral and pulmonary artery smooth muscle cells. *American journal of physiology Cell physiology*. 2013; 304:C780–789. DOI: 10.1152/ajpcell.00006.2012 [PubMed: 23426969]
95. Zhou Y, Schopperle WM, Murrey H, Jaramillo A, Dagan D, Griffith LC, Levitan IB. A dynamically regulated 14-3-3, Slob, and Slowpoke potassium channel complex in *Drosophila* presynaptic nerve terminals. *Neuron*. 1999; 22:809–818. [PubMed: 10230800]

Fig. 1. Dilatation by lithocholate, a BK $\beta 1$ ligand, is highest in basilar and coronary arteries
 (a) Representative arterial diameter trace from rat MCA pressurized *in vitro*. After development of myogenic tone, the artery was probed with 60 mM KCl to test maximal contraction. After KCl washout, the artery chamber was perfused with PSS that contained vehicle (DMSO, 0.1% v/v). Then, artery was probed with 45 μ M LCA, which constitutes EC_{50} for LCA activation of $\beta 1$ -containing BK channels [15]. Vertical lines underscore the beginning of perfusion with any given solution. The shadowed area shows the AUC corresponding vasodilation by LCA, which is fully reversible. An identical protocol was used with ACA, PCA, BA, MA and CA. (b) Bar graph demonstrating that LCA-induced dilation of BA and CA is drastically larger that evoked on ACA, MCA, PCA or MA; $n=3-7$, *Different from BA, $P<0.05$; ACA, anterior cerebral artery; MCA, middle cerebral artery; PCA, posterior cerebral artery; BA, basilar artery; MA, mesenteric artery; CA, coronary artery; LCA, lithocholate.

Fig. 2.

Representative BK channel recordings at single-channel resolution obtained from inside-out membrane patches from rat ACA (a), MCA (b), PCA (c), BA (d), MA (e) and CA (f) myocytes. These records show that LCA-induced increase in channel steady-state activity (NP_o) is larger in BA and CA myocytes than in myocytes isolated from another arteries. $V_m = -40$ mV; $Ca^{2+}_i = 3$ μ M. Recordings were obtained before (top trace on each panel), during (middle trace) and after (bottom trace) patch exposure to 45 μ M LCA. Arrows indicate the baseline (all channels closed); ACA, anterior cerebral artery; MCA, middle cerebral artery; PCA, posterior cerebral artery; BA, basilar artery; MA, mesenteric artery; CA, coronary artery; LCA, lithocholate.

Fig. 3. Lithocholate-induced increase in BK channel activity is significantly larger in myocytes from coronary and basilar than in myocytes from another arteries

Bar graph showing averaged NP_o ratios in presence and absence of $45 \mu\text{M}$ LCA from ACA, MCA, PCA, BA, MA and CA myocyte BK channels; $n=5-10$, *Different from BA, $P<0.05$; ACA, anterior cerebral artery; MCA, middle cerebral artery; PCA, posterior cerebral artery; BA, basilar artery; MA, mesenteric artery; CA, coronary artery; LCA, lithocholate; N, number of functional channels present in the membrane patch; Po, single channel open probability.

Fig. 4. Steady-state BK channel activity in basilar and coronary artery is higher than in myocytes isolated from other intracranial arteries and from mesenteric artery

(a) Averaged $P_o/P_{o\max}$ -V curves of BK channels in myocytes isolated from MA, CA and the different branches of the Willis' circle. (b) Bar graph showing averaged V_{half} values. $N=3-4$; * $p<0.05$, different from myocytes from BA. ACA, anterior cerebral artery; MCA, middle cerebral artery; PCA, posterior cerebral artery; BA, basilar artery; MA, mesenteric artery; CA, coronary artery; P_o , single channel open probability.

Fig. 5. Representative images of individual myocytes isolated from different cerebral arteries. Myocytes were subjected to immunofluorescence staining against (a) BK channel-forming α (green) and (b) accessory $\beta 1$ (red) subunits. ACA, anterior cerebral artery; MCA, middle cerebral artery; PCA, posterior cerebral artery; BA, basilar artery; MA, mesenteric artery; CA, coronary artery.

Fig. 6. BK α protein levels in myocyte membranes are similar across the different arteries whereas BK β 1 subunit levels in the plasmalemma-subplasmalemma region are significantly higher in basilar and coronary arteries than in other arteries

Averaged fluorescence intensity (fold difference *compared to MCA*) of BK α (a) and BK β 1 (b) subunit-associated signal in myocytes from intracranial arteries, MA and CA. Each bar value represents an average of 22–74 myocytes isolated from 2–3 different rats for each vascular bed. * $p < 0.05$, when compared to expression levels in BA myocytes. ACA, anterior cerebral artery; MCA, middle cerebral artery; PCA, posterior cerebral artery; BA, basilar artery; MA, mesenteric artery; CA, coronary artery.

Fig. 7. BK β 1 mRNA transcript level is higher in basilar and coronary arteries than in other arteries

Bar graph showing BK β 1 mRNA levels measured using quantitative real-time RT-PCR across the different intracranial arteries, MA and CA. mRNA level in a given artery was normalized to expression of an endogenous control gene, i.e., Tata Binding Protein (TBP) in the same artery. Then, normalized mRNA expression levels in any given artery were further normalized to mRNA expression levels in MCA. $n=8-11$; $*p<0.05$, different from expression level in BA. ACA, anterior cerebral artery; MCA, middle cerebral artery; PCA, posterior cerebral artery; BA, secondary branches of basilar artery; MA, quaternary branches of mesenteric artery; CA, coronary artery.

Fig. 8. Overexpression of BK β 1 protein in middle cerebral artery significantly increases the dilatory response of this artery to lithocholate

(a) Representative Western blots showing expression of BK β 1 protein in arteries transfected with “empty” vector (pcDNA3; left lane) or pcDNA3+BK- β 1 cDNA insert (right lane). Actin levels were used as control. (b) Electroporation of MCA with pcDNA3+cDNA BK β 1 leads to significantly higher levels of BK β 1 protein when compared to control (pcDNA3). (c) Diameter trace from de-endothelialized, pressurized rat MCA transfected with “empty” vector (pcDNA3). (d) Diameter trace from de-endothelialized, pressurized MCA transfected with BK β 1 cDNA. (e) Averaged data showing that in rat MCA transfected with BK β 1 cDNA, application of 45 μ M LCA evokes an arterial dilation that is significantly higher than that from arteries transfected with empty vector (pcDNA3). $n=4-5$; * $P<0.05$, when

compared to arteries transfected with pcDNA3 alone; MCA, middle cerebral artery; LCA, lithocholate.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

TABLE 1

Transcripts	Primers
<i>KCNMB1</i>	
Forward	5'-catgcctttgggtcaatga-3'
Reverse	5'-tcctggggatataggagcact-3'