Molecular Pharmacology Fast Forward. Published on April 27, 2007 as doi:10.1124/mol.107.034330

MOL 34330

β_1 (*KCNMB1*)-subunits mediate lithocholate activation of BK channels and dilation in small, resistance arteries

Anna N. Bukiya, Jianxi Liu, Ligia Toro, and Alex M. Dopico*

Department of Pharmacology, University of Tennessee Health Science Center,

Memphis, TN 38163

AFFILIATION: Department of Pharmacology, University of Tennessee Health Science Center,

Memphis, TN 38163 (A.B., J.L., and A.M.D.)

Department of Anesthesiology, University of California Los Angeles,

Los Angeles, CA 90095 (L.T.)

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

MOL 34330

RUNNING TITLE: Lithocholate activates BK channels and vasodilates *via KCNMB1*

CORRESPONDING AUTHOR: Alex Dopico, MD, PhD
The Univ. Tennessee Hlth. Sci. Ctr.
Dept. Pharmacology
874 Union Ave.
Memphis, TN 38163
Phone: 901-448-3822
Fax: 901-448-1695
Email: adopico@utmem.edu

TEXT PAGES: 30

TABLES: 1

FIGURES: 6

SUPPL. FIGURES: 2

REFERENCES: 40

WORDS IN ABSTRACT: 246

WORDS IN INTRODUCTION: 567

WORDS IN DISCUSSION: 1,600

ABBREVIATIONS: LC, lithocholate; BK, large-conductance, Ca^{2+}-activated K^+; PSS, physiological saline solution; Ibtx, iberiotoxin; DM, dissociation medium; I/O, inside-out; O/O, outside-out; N, number of functional channels in the patch; P_o, open channel probability; DMSO, dimethyl sulfoxide; CMC, critical micellar concentration; CBF, cerebral blood flow; K_V, voltage-gated K^+; 4-AP, 4-aminopyridine; z, effective valence; DHS-1, dehydrosoyasaponin-1

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Among the nongenomic effects of steroids, control of vasomotion has received increasing attention. Lithocholate (LC) and other physiologically relevant cholane-derived steroids cause vasodilation, yet the molecular targets and mechanisms underlying this action remain largely unknown. We demonstrate that LC (45 μM) reversibly increases the diameter of pressurized resistance cerebral arteries by ~10%, which would result in ~30% increase in cerebral blood flow. LC action is independent of endothelial integrity, prevented by 55 nM iberiotoxin, and unmodified by 0.8 mM 4-aminopyridine, indicating that LC causes vasodilation *via* myocyte BK channels. Indeed, LC activates BK channels in isolated myocytes through a destabilization of channel long-closed states without modifying unitary conductance. LC channel activation occurs within a wide voltage range and at Ca^{2+} concentrations reached in the myocyte whether at rest or during contraction. Channel accessory β_1-subunits, which are predominant in smooth muscle, are necessary for LC to modify channel activity. In contrast, β_4-subunits, which are predominant in neuronal tissues, fail to evoke LC sensitivity. LC activation of cbv1+β_1 and native BK channels display identical characteristics, including EC_{50} (46 μM) and E_{max} (≈300 μM), strongly suggesting that the cbv1+β_1 complex is necessary and sufficient to evoke LC action. Finally, intact arteries from β_1-subunit knockout mice fail to relax in response to LC, although they are able to respond to other vasoactive agents. This study pinpoints the BK β_1-subunit as the molecule that senses LC, which results in myocyte BK channel activation and, thus, endothelial-independent relaxation of small, resistance arteries.

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

Acute, nongenomic effects of steroids are receiving increasing attention as, in some cases, they have led to discovery of novel receptor proteins (Watson and Gametchu, 2003), with the possible development of steroidal analogs that have therapeutic use. Among these nongenomic effects, it is particularly noticeable that several physiologically relevant steroids have vasoactive properties. While vascular reactivity and tone are regulated by neuronal, endocrine, and endothelial factors, vascular tone and contractility are ultimately determined by signaling molecules and ion channels that operate in the vascular myocyte itself. Some steroids, such as estradiol (Valverde et al., 1999), xenoestrogens (Dick and Sanders, 2001; Perez, 2005), and glucocorticoids (Lovell et al., 2004), modify ion channel function *via* direct interactions with ion channel proteins. Others, such as aldosterone (Asher et al., 1996) and estradiol (White et al., 2002), target signaling systems that, in turn, modulate ion channels. Some others, such as cholesterol (Bolotina et al., 1989), alter the physicochemical properties of the lipid microenvironment in which channel proteins are embedded, with modification of channel conformation and function.

Bile acids (cholane-derived steroids) attenuate vascular reactivity *in vitro* and reduce systemic blood pressure *in vivo* (Pak and Lee, 1993; Bomzon and Ljubuncic, 1995). Furthermore, a spillover of bile acids from the portal to the systemic circulation is responsible for the systemic hypotension observed in patients with liver damage and/or significant porto-systemic circulatory shunt. In some of these subjects, systemic circulating levels of bile acids may reach more than 100 μM (Ostrow, 1993). It has even been speculated that bile acids may serve as endogenous vasodilators (Bomzon and Ljubuncic, 1995), and bile acid relaxation of vascular smooth muscle appears to be secondary to steroid actions on the smooth muscle itself (Ljubuncic et al., 2000). Remarkably, the vasoactive properties of bile acids have not been tested in small, resistance arteries that develop myogenic tone, these arteries being critical to

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

determining pressure and blood flow. Moreover, the molecular targets and mechanisms by which bile acids cause vasodilation remain largely unknown.

In a previous study, we found that lithocholate (LC) and other naturally occurring bile acids, as well as LC synthetic analogs, increase the activity of large-conductance, Ca^{2+}-activated K^+ (BK) channels in myocytes isolated from large arteries (Dopico et al., 2002). Since BK channel activation is a mechanism that opposes constriction and attenuates arterial tone (Jaggar et al., 2000), our previous finding raised the speculation that LC-mediated activation of BK channels in small, resistance artery smooth muscle is responsible for arterial tone modification by this steroid.

Here, we demonstrate that acute application of LC readily and reversibly increases the activity of native BK channels freshly isolated from small, resistance arteries. Vascular smooth muscle BK channels are made of channel-forming α (*KCNMA1*)- and regulatory β_1 (*KCNMB1*)-subunits (Orio et al., 2002). In contrast, BK $\alpha+\beta_4$ (*KCNMB4*)-subunits are predominant in neuronal tissues (Brenner et al., 2000a; Meera et al., 2000). After cloning α-subunits (termed "cbv1"; AY330293) from myocytes freshly isolated from rat resistance cerebral arteries, we used recombinant BK channels to demonstrate that the channel β_1-subunit acts as the LC sensor. In contrast, β_4-subunits fail to render BK channels sensitive to LC. Moreover, pharmacological block of BK (but not other K_V channels that also control myocyte tone) or genetic ablation of BK β_1-subunits prevents LC from dilating small, resistance arteries. Finally, endothelial removal does not modify LC-induced vasodilation, raising the possibility that LC-based compounds could be used as effective endothelium-independent vasodilators.

Materials and Methods

Artery Diameter Measurement. Middle cerebral arteries were isolated from adult male Sprague-Dawley rats (≈250 g) or 8- to 12-week-old β_1-knockout and C57BL/6 control mice. Rats

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

and 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. Pressurization of arteries was performed as described (Liu et al., 2004). Endothelium was removed by passing an air bubble into the vessel lumen for 90 sec. Diameter changes were monitored through an inverted microscope (Nikon Eclipse TS100, Nikon Corp., Tokyo, Japan), recorded on camera (Sanyo VCB-3512T, Sanyo Electric Corp., Japan), and transferred to a computer. Diameter data were acquired and analyzed using IonWizard 4.4 software (IonOptics Corp., Milton, MA).

Pressurized arteries were extraluminally perfused with physiological saline solution (PSS) (Liu et al., 2004) at a constant rate of 3.75 ml/min using a peristaltic pump Dynamax RP-1 (Rainin Instrument, Inc., Oakland, CA). At this rate, complete washout of the iberiotoxin (Ibtx) effect required >45 min. To keep basal tone under steady behavior, we shortened this period by increasing flow rate ~3 times during washout of Ibtx, which sometimes evoked a flow-induced dilation (Fig. 1A). Equal volumes (25 ml) of vehicle- *vs.* LC-containing solutions were applied at equal, constant rate (see above) to the pressurized arterial segment in the chamber *via* a gravity system. Drugs were dissolved to make stock solutions (see Chemicals) and diluted in PSS to final concentration.

Myocyte Isolation. Basilar and middle cerebral arteries were dissected out from each brain under a stereozoom microscope (Nikon C-PS, Tokio, Japan) and placed into ice-cold "dissociation medium" (DM) (mM): 0.16 $CaCl_2$, 0.49 EDTA, 10 HEPES, 5 KCl, 0.5 KH_2PO_4, 2 $MgCl_2$, 110 NaCl, 0.5 NaH_2PO_4, 10 $NaHCO_3$, 0.02 phenol red, 10 taurine, 10 glucose. Each artery was cut into 1-2 mm long rings (~30 rings/experiment). Rings were put in 3 ml DM containing 0.03% papain, 0.05% bovine serum albumin (BSA) and 0.004% dithiothreitol (DTT) for 15 min at 37 ºC in a polypropylene centrifuge tube, and then incubated in a shaking water bath at 37 ºC and 60 oscillations/min. for 15 min. The prepartion was then centrifuged several

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

times as described (Liu et al., 2004). After the final centrifugation, the supernatant was discarded, and the pellet resuspended in 3 ml of DM containing 0.06% soybean trypsin inhibitor. Finally, the tissue 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 40X objective) that could be easily identified under microscope (Olympus IX-70; Olympus America, Woodbury, NY). The cell suspension was stored in ice-cold DM containing 0.06% BSA, and the cells were used for patch-clamping up to 4 h after isolation.

cRNA Preparation and Injection into *Xenopus* Oocytes. Full-length cDNA coding for cbv1-subunits was cloned from rat cerebral artery myocytes by PCR and ligated to the PCR-XL-TOPO cloning vector (Invitrogen Corp., Carlsbad, CA) (Jaggar et al., 2005). cDNA coding for cbv1-subunits was cleaved from the cloning vector by BamHI (Invitrogen Corp., Carlsbad, CA) and XhoI (Promega, Madison, WI) and directly inserted into the pOX vector for expression in *Xenopus* oocytes. pOX-cbv1 was linearized with NotI (Promega, Madison, WI) and transcribed *in vitro* using T3 polymerase. $Beta_1$-subunit cDNA inserted into the EcoR I/Sal I sites of the pCI-neo expression vector was linearized with NotI and transcribed *in vitro* using T7 polymerase. $Beta_4$-subunit cDNA inserted into the pOx vector was linearized by NotI and transcribed using T3 polymerase. The mMessage-mMachine kit (Ambion Inc., Austin, TX) was used for transcription. The pOX vector and the cDNA coding for β_1-subunits were generous gifts from Aguan Wei (Washington University, Saint Louis, MO) and Maria Garcia (Merck Research Laboratories, Whitehouse Station, NJ).

Oocytes were removed from *Xenopus laevis* and prepared as described (Dopico et al., 1998). cRNA was dissolved in diethyl polycarbonate-treated water at 5 (cbv1) and 15 (β_1 or β_4) ng/μl; 1-μl aliquots were stored at -70°C. Cbv1 cRNA was injected alone (2.5 ng/μl) or coinjected with either β_1 or β_4 (7.5 ng/μl) cRNAs, giving molar ratios ≥6:1 (β:α). cRNA injection (23 nl/oocyte)

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

was conducted using a modified micropipette (Drummond Scientific Co., Broomall, PA). The interval between injection and patch-clamp recordings was 48-72 h.

Electrophysiology. Oocytes were prepared for patch-clamp recordings as described (Dopico et al., 1998). Single-channel and macroscopic currents were recorded from inside-out (I/O) or outside-out (O/O) patches. For experiments with oocytes, both bath and electrode solutions contained (mM) 135 K^+ gluconate, 5 EGTA, 1 $MgCl_2$, 15 HEPES, 10 glucose, pH 7.35. For experiments with myocytes, KCl substituted for K^+ gluconate. In all experiments, free Ca^{2+} in solution was adjusted to the desired value by adding $CaCl_2$. In most studies, free Ca^{2+} in electrode solution=10 μM. In O/O studies with 17β-estradiol, however, free Ca^{2+} in the electrode solution=0.3 μM. Nominal free Ca^{2+} was calculated with MaxChelator Sliders (C. Patton, Stanford University, CA) and validated experimentally using Ca^{2+}-selective electrodes (Corning Incorporated Science Products Division, Corning, NY).

Patch-recording electrodes were made as described (Dopico et al., 1998). Immediately before recording, the tip of each electrode was fire-polished on a microforge WPI MF-200 (World Precision Instruments, Inc., Sarasota, FL) to give resistances of 5-9 MΩ when filled with solution. An agar bridge with gluconate or Cl^- as the main anion (for oocyte and myocyte experiments, respectively) was used as ground electrode. After excision from the cell, the membrane patch was exposed to a stream of bath solution containing each agent at final concentration. Solutions were applied onto the patches using a pressurized system DAD12 (ALA Scientific Instruments, New York, NY) *via* a micropipette tip with an internal diameter of 100 μm. Experiments were carried out at room temperature (21°C).

Currents were recorded using an EPC8 amplifier (HEKA Electronics, Lambrecht/Pfalz, Germany) at 1 kHz using a low-pass, eight-pole Bessel filter 902LPF (Frequency Devices, Haverhill, MA). Data were digitized at 5 kHz using a Digidata 1320A A/D converter and

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

pCLAMP 8.0 (Molecular Devices, Union City, CA). For macropatch recordings, G/Gmax-V data were fitted to the Boltzmann function: $G(V)=G_{max}/(1 + e^{(-V + V_{0.5})/k})$

Using the slope (k) of the G/G_{max} *vs.* V plots, z (i.e., 1/k) was calculated as 1/k = RT/F, where R, T, and F have their usual meaning. As an index of channel steady-state activity, we used the product of the number of channels in the patch (N) and the channel open probability (P_o). NP_o was obtained from all-points amplitude histograms from ≥30 sec of continuous recording under each experimental condition.

Chemicals. All chemicals were purchased from Sigma (St. Louis, MO), with the exception of 5β-cholanic acid 3α-ol (LC) (Steraloids, Inc., Newport, RI) and Ibtx (Alomone Labs Ltd., Jerusalem, Israel). On the day of the experiment, an LC stock solution (333 mM) in dimethyl sulfoxide (DMSO) was freshly prepared by sonication for 5 min. For arterial tone experiments, the LC stock was diluted 1/10 in DMSO and further diluted in PSS to render final LC concentration. Solution containing vehicle (0.1% DMSO; V/V) was used as control perfusion. For electrophysiological recordings, the LC stock solution was diluted 1/10 in 95% ethanol and further diluted with bath solution to render final LC concentration (3-1,000 μM). The DMSO/ethanol vehicle (≤0.1/≤0.86% final concentrations) in bath solution was used as control.

Data Analysis. Artery diameter response to each compound is shown as a percentage of the diameter obtained before compound application. Arterial diameter and electrophysiological data were analyzed with IonWizard 4.4 (IonOptics Corp., Milton, MA) and pCLAMP 8.0 (Molecular Devices, Union City, CA). Further analysis, plotting, and fitting were conducted using Origin 7.0 (Originlab Corp., Northampton, MA) and InStat 3.0 (GraphPad Software Inc., San Diego, CA). Statistical analysis was conducted using either one-way ANOVA and Bonferroni's multiple comparison test or paired Student's *t*-test; significance was set at $p<0.05$. Data are expressed as mean±SEM; n=number of patches/arteries.

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Lithocholate Dilates Small, Resistance Arteries *via* BK Channels. After myogenic tone development at 60 mm Hg, intact arteries reached a diameter of 154.4±5.2 μm (n=17). Maximal contraction and dilation were checked by perfusing the vessel with 60 mM KCl at the beginning, and Ca^{2+}-free solution at the end of each experiment (Fig. 1A,B). In all cases (n=17), application of 45 μM LC, that is, concentrations well below LC's critical micellar concentration (CMC) (≥1 mM under our recording conditions) (Roda et al., 1995) caused a significant increase in peak arterial diameter: +6% *on top* of a transient increase in diameter caused by vehicle-containing solution (Figs. 1A-C). LC-induced dilation was not only larger than that caused by vehicle but also more sustained (≈2.8 times longer); for example, by the time the vehicle effect had totally vanished, LC-induced dilation still represented 109.9±1.7% of the initial arterial diameter determined before any compound application (n=17; $p<0.01$). The differential vasodilation caused by LC *vs.* vehicle is most evident from the area under the curve values (integrals) corresponding to the change in diameter as a function of time: 18,369± 3,964 *vs*. 10,262 ±2,574 ($p<0.01$) (Table 1). Notably, the net increase in cerebral artery diameter caused by LC over pre-LC values (+9.9%) is expected to cause a marked increase (~30%) in cerebral blood flow (CBF), because changes in artery diameter are related to changes in CBF by a factor of ~3 (Gourley and Heistad, 1984).

Rat cerebral artery diameter is critically controlled by myocyte BK channel activity (Jaggar et al., 2000). Since these channels are selectively blocked by nanomolar concentrations of Ibtx (Liu et al., 2004), we used this peptide to determine any possible contribution of BK channels to LC-dilation. As expected, bath application of 55 nM Ibtx caused a robust decrease in the diameter of intact arteries (-11.8±3.4%) (n=4) (Fig. 1A). Remarkably, LC dilation was completely lost when the steroid was applied on top of Ibtx (Fig. 1A,C). In the presence of Ibtx, LC caused some reduction in diameter (-3.4%), which could be related to the well-known

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

increase in cytosolic Ca^{2+} caused by bile acids (Thibault and Ballet, 1993). In brief, our data indicate that LC fails to dilate small, resistance arteries when BK channels are specifically blocked.

Cerebrovascular smooth muscle tone is also controlled by voltage-gated K^+ channels (K_V) other than BK (Faraci and Sobey, 1998). To determine the selectivity of BK channel involvement in LC dilation, we evaluated LC action in the presence of 4-aminopyridine (4-AP) which, at sub- to low millimolar concentrations, blocks most K_V but not BK channels in rat cerebral arteries (Liu et al., 2004). Applying 0.8 mM 4-AP caused an immediate decrease in diameter (-9.7±2.3%, n=4) (Fig. 1B). In contrast to the Ibtx results, the change in peak diameter caused by LC in the presence of 4-AP was identical to that determined in the absence of K_V blocker (+6% over pre-LC values; Fig. 1C). While we cannot rule out some contribution of K_V channels other than BK to LC dilation, our results indicate that K_V channels other than BK do not play a major role in LC dilation of pressurized small, resistance cerebral arteries. Furthermore, 45 μM LC on top of 4-AP almost totally reverted the vasoconstriction caused by the K_V channel blocker (Fig. 1B), underscoring the effectiveness of BK channel-targeting by LC in reversing cerebrovascular constriction driven by voltage-dependent mechanisms. In contrast to LC dilation, the small and transient increase in diameter caused by vehicle was unmodified by Ibtx (Fig. 1A) but somewhat decreased by 4-AP (Fig. 1B). The mechanism(s) involved in the transient dilation evoked by vehicle is out of the scope of this study. Nevertheless, the differences in time-course and magnitude (Table 1), together with their differential modulation by selective channel blockers, clearly indicate that LC and vehicle dilation of cerebral arteries are mediated by different ionic mechanisms, the former *via* BK channels.

Finally, to rule out that endothelial factor(s) could be mediating or, at least, modulating LC-induced dilation, we studied LC action in de-endothelized arteries and compared it to that in intact vessels. LC-induced dilation is similar in intact *vs.* endothelium-denuded arteries (n=5).

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

The presence of a functional endothelium was assessed by vascular responses to endothelium-dependent (acetylcholine; 10 μM) and independent (sodium nitroprusside; 10 μM) vasodilators. Indeed, while vasodilation in response to acetylcholine was lost (n=4), sodium nitroprusside-induced dilation was fully preserved in de-endothelized arteries (n=5) (Fig. 1D). Notably, LC increase in diameter of de-endothelized arteries was not significantly different from that of intact arteries (Fig. 1D). Thus, LC-induced dilation of small, resistance cerebral arteries is independent of a functional endothelium. Collectively, data shown in Fig. 1 suggest that LC dilation of small cerebral arteries is due to LC targeting of *myocyte* BK channels.

Lithocholate Directly Activates Myocyte BK Channels *via* the Channel β_1-subunit. To determine whether LC directly targets BK channels in cerebral artery myocytes, we studied drug action on channel activity by using I/O patches with the membrane potential and free Ca^{2+}_i set at values (-40 to -30 mV and 3 μM) similar to those obtained in cerebrovascular myocytes during contraction (Knot and Nelson, 1998; Perez et al., 2001). After excision, the patch was exposed to vehicle-containing solution, and BK NP_o was recorded for no less than 1 minute. Then, applying LC-containing (1-1,000 μM) solution reversibly increased NP_o in a concentration-dependent fashion: EC_{50}=46±6 μM, E_{max}~300 μM (Figs. 2A,B). At E_{max}, NP_o reached 350% of control, this ceiling remaining steady up to 1 mM LC. Concentrations above 1 mM (i.e., close to the CMC for LC under our recording conditions) (Roda et al., 1995) systematically resulted in loss of gigaseals, likely due to a micelle-mediated detergent effect. Thus, LC maximally increases BK channel activity at aqueous concentrations in which LC monomers predominate, as opposed to a detergent action on the membrane due to micelle formation in solution. LC increase in NP_o was observed in membrane patches that were excised from the myocyte >5 min before applying LC under continuous bath perfusion in the absence of nucleotides. Therefore, LC action does not require cell integrity or the continuous presence of cytosolic messengers. Rather, it is due to a

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

direct interaction between the steroid and the BK channel complex itself and/or its immediate proteolipid environment.

To determine which subunit of the channel complex is involved in sensing LC with eventual increase in NP_o, we performed electrophysiological recordings in I/O patches from *Xenopus* oocytes expressing either homomeric cbv1 or heteromeric cbv1+β_1 channels under identical conditions (to evoke measurable levels of P_o within a sec-min time frame in the absence of β_1-subunits, these studies were conducted at Ca^{2+}_i=10 μM, at either positive or negative V_m (+20 or -20 mV); considering that LC effect on BK channel NP_o is voltage-independent (see Dopico et al., 2002 and below), data obtained at both voltages were pooled. Cbv1-subunits expressed in oocytes rendered macro- and microscopic currents that showed all major biophysical and pharmacological features of BK currents (Jaggar et al., 2005). The presence of functional β_1-subunits was confirmed by macroscopic currents characteristics (Brenner et al., 2000a) (slower activation kinetics, increased apparent Ca^{2+}-sensitivity with a shift in $V_{0.5}$ of ~20 mV towards negative potentials) and channel activation by bath application of 10 μM 17β-estradiol to the extracellular surface of O/O patches (Valverde et al., 1999) (Suppl. Figs. 1A,B and 2B).

In contrast to the results obtained with native BK channels in cerebrovascular myocytes, application of LC as high as 150 μM (on top of vehicle) to the internal side of I/O patches failed to activate homomeric cbv1 channels, with average NP_o reaching 112±13% of control ($p>0.05$; n=4) (Figs. 3A,C). Thus, LC activation of cerebrovascular BK channels requires the presence of β_1-subunits and/or some other component of the myocyte membrane that is missing in the heterologous expression system. As found with native BK channels, however, LC (3-300 μM) caused a reversible and concentration-dependent increase in NP_o of heteromeric cbv1+β_1 channels (Figs. 3B,C), with EC_{50}=43.5±4.7 μM and E_{max}~300 μM, at which NP_o reached 290±45% of control. These values are practically identical to those of native BK channels (see above), indicating that differences in composition/organization between rat cerebrovascular

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

myocyte and *Xenopus* oocyte membranes are not critical in LC action on BK channels. The identical LC responses of native cerebrovascular BK and cbv1+β_1 channels appear to indicate the involvement of a common target(s) mediating LC action in these two systems, possibly the β_1-subunit itself.

A Hill-like plot for LC-activation of cbv1+β_1 channels renders a slope (*apparent* Hill coefficient) of 1.3 (Fig. 3D), which suggests the involvement of at least two "sites" in the cbv1+β_1 complex for LC to increase NP_o. An increase in the number of channels (N) might contribute to the overall increase in NP_o caused by LC. Data from patches where N=1 (Fig. 4A), however, show an increase in P_o that is similar to the increase in NP_o in patches containing an unknown N. Thus, LC action on BK steady-state activity appears to be solely determined by an increase in P_o. Given the apparent Hill coefficient of 1.3, the increase in P_o appears to require the interaction of at least two LC molecules with the β_1-subunits of the channel complex.

From the channel dwell-time distributions in patches (n=2) where N=1 (Fig. 4A), we calculated both open and mean closed times (Dopico et al., 1998). Both distributions could be well-fitted with double exponential functions, indicating the existence of at least two open and two closed states. Lithocholate increased the channel mean open time, which reached 137% of control. This enhancement resulted from an LC-induced increase in the average duration of both short and long open channel events, with an accompanying mild shift in the open channel distribution towards longer openings; the long open state(s) accounted for 49% and 57% of total open events in vehicle and LC, respectively (Figs. 4A,B). In addition, LC drastically decreased the channel mean closed time, which reached 41% of control. This reduction was primarily caused by a robust reduction in the average duration of channel long closed events, and also a shift towards briefer closures; the long close state(s) accounted for 44% and 34% of total close events in vehicle and LC, respectively (Figs. 4A,B). In brief, the steroid-induced increase in channel P_o results primarily from LC-induced destabilization of channel long closed states,

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

eventually reducing by more than half the channel mean closed time. These changes in channel kinetics with consequent increase in P_o occurred in the absence of significant change in unitary conductance: 228.6±3.7 vs. 234.0±4.6 pS in symmetric 135 mM K^+ (n=4; NS). Thus, LC modification of BK channel function is limited to modification of channel gating (see Discussion).

To determine whether LC increase in BK P_o is selectively mediated by the β-subunit type (β_1) that is predominant in smooth muscle or could be mediated by other channel accessory-subunits, we tested LC action on cbv1+β_4 channels. When coexpressed with α-subunits, β_4-subunits introduce a hyperpolarizing shift in $V_{0.5}$ similar to that caused by α+β_1 coexpression. In addition, β_4-subunits render the BK complex relatively resistant to Ibtx (Meera et al., 2000). This phenotype was confirmed in our study (Suppl. Fig. 2A,B). Under conditions identical to those used with cbv1 and cbv1+β_1 channels, cbv1+β_4 channels were consistently refractory to LC action (8/8 patches), even when tested at concentrations (150 μM) that were close to E_{max} in both cbv1+β_1 and native BK channels (Fig. 3E,F); cbv1+β_4 NP_o reached 109±11% of control (NS, also not significantly different from LC action on cbv1 homomeric channels). Therefore, β_1- but not β_4-subunits confer LC sensitivity to cerebrovascular BK channels.

Lithocholate Effectively Activates BK Channels within Physiological Ranges of Ca^{2+}_i and Membrane Voltage. β_1-subunits modulate both Ca^{2+}-dependent and -independent channel gating, resulting in an increase in the *apparent* Ca^{2+} sensitivity of the channel. This effect is more pronounced at Ca^{2+}_i that effectively increases P_o (Meera et al., 1996; Nimigean and Magleby, 2000). On the other hand, the lateral chain of LC contains a carboxyl that is ionized at physiological pH, raising the possibility that LC action could be modified by transmembrane voltage. Thus, we explored the Ca^{2+}- and voltage-dependence of LC action on cbv1+β_1 channel P_o by using a wide voltage range (±150 mV) and Ca^{2+}_i levels that expanded those in the myocyte under physiological conditions (0.15-0.3 μM at rest; up to 10-30 μM in the vicinity of BK

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

channels during contraction) (Perez et al., 2001; Liu et al., 2004). Even at non-physiological, very positive voltages (+80 mV), LC potentiation of BK NP_o was unnoticeable when recorded in solutions having zero Ca^{2+} added plus 10 mM EGTA to chelate trace amounts of the divalent (n=3) (data not shown). This is consistent with LC modulating channel gating *via* a β_1-mediated mechanism, as at "zero" or subactivatory Ca^{2+}_i, β_1-subunit modification of gating does not translate into an evident change in overall P_o (Nimigean and Magleby, 2000). Furthermore, LC-activation (as a percentage of NP_o in vehicle) increased with Ca^{2+}_i: from 139.9±32.9 (n=4; $p<0.05$) at 0.1 μM Ca^{2+} to a maximal effect of 244.1±58.9% (n=3; $p<0.01$) at 1 μM Ca^{2+}. This maximum remained steady within the 1 to 10 μM Ca^{2+}_i range (n=16), to decrease with higher Ca^{2+}_i (e.g., at 30 μM, NP_o in LC reached 172.5±9.5% of control; $p<0.05$, n=3) (Fig. 5A). These data demonstrate that LC activates BK channels within a Ca^{2+} range that spans from resting levels to those reached during myocyte contraction. Remarkably, LC activation is most effective at Ca^{2+} levels reached near the BK channel during cerebral artery myocyte contraction (Perez et al., 2001).

Next, we evaluated LC action on cbv1+β_1-mediated currents as a function of applied voltage, exposing I/O macropatches to Ca^{2+}_i at which LC activation of BK channels is robust: 0.3, 3, and 10 μM Ca^{2+}_i. From G/Gmax *vs.* V_m plots fitted to a Boltzmann relationship, we obtained $V_{0.5}$=101.9±1.2 (n=3), 74±10 (n=3), and 32.3±11.2 mV (n=5), respectively. At every Ca^{2+}_i tested, LC (150 μM) shifted the $V_{0.5}$ by ≈-17.7 mV (Fig. 5B) without changing the slope of the plot. Thus, at any constant Ca^{2+}_i, the effective valence (i.e., an index of the minimum number of elementary charges that cross the electric field to gate the channel) (z) was similar in the absence or presence of LC (e.g., at 10 μM free Ca^{2+}_i: z=1.24±0.29 *vs.* 1.26±0.2). These data suggest that LC does not interfere with the voltage-sensing process of channel gating. The lack of LC effect on *z* is also consistent with a β_1-mediated action on channel gating (Brenner et al., 2000a).

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

Together, the data show that LC is an effective activator of BK channels *via* their β_1-subunits at physiologically relevant Ca^{2+}_i and voltages.

Lithocholate Fails to Induce Cerebrovascular Dilation in β_1-Knockout Mice. To determine the impact of LC targeting of BK β_1-subunits on organ function, we evaluated LC action on the arterial diameter of pressurized cerebral arteries from β_1-knockout *vs. wt* C57BL/6 mice (controls). To verify the presence of functional β_1-subunit-containing BK channels in controls, we tested artery diameter sensitivity to block by 55 nM Ibtx, as done with rat arteries (Fig. 1A). In control mice, Ibtx caused a significant vasoconstriction within 15 min of application (up to -8.7±4.2% decrease from initial diameter; $p<0.01$; n=4) (Fig. 6A,B). As reported (Brenner et al., 2000b), Ibtx decrease in diameter was largely attenuated in arteries from β_1-knockout mice (-2.25±0.44%; different from vasoconstriction in *wt* mice, $p<0.05$; n=4) (Figs. 6A,B).

The mild and transient vehicle dilation found in rat arteries was also observed in mouse arteries. Consistent with results obtained in rat arteries showing the lack of Ibtx modulation of vehicle dilation (Fig. 1A), genetic ablation of β_1-subunits failed to modify vehicle action (Fig. 6A), buttressing the idea that this mild and transient dilation does not involve BK channels.

More important, as found with rat cerebral arteries, 45 μM LC caused a sustained yet fully reversible increase in diameter of *wt* mouse cerebral arteries (+4.4±0.9% from initial diameter; $p<0.01$; n=7). In sharp contrast, LC consistently failed to dilate arteries from β_1-knockout mice (n=5) (Fig. 6A,B), indicating that in intact cerebral arteries the presence of BK β_1-subunits is crucial for LC dilation.

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

We have demonstrated for the first time that LC is an effective dilator of pressurized, resistance arteries, this relaxation being endothelium-independent. LC-induced vasodilation is due to selective targeting of myocyte BK channel function. While a variety of ion channels other than BK contribute to regulate cerebrovascular tone (Faraci and Sobey, 1998; Dietrich et al., 2005), making them putative targets of LC effect on vasomotion, the fact that genetic ablation of *KCNMB1* (but not of *KCNMB4*) or selective pharmacological block of BK (but not other K_V) channels suppresses LC-mediated cerebrovascular dilation clearly indicates that the BK channel β_1-subunit is the molecular effector of LC-induced cerebrovascular dilation. Lithocholate targeting of BK channels results in a reversible increase in P_o due to several LC actions on channel dwell-times, with destabilization of the channel long closed states being predominant. Lithocholate action on P_o is evident within wide voltage and Ca^{2+}_i ranges, which include values measured in cerebrovascular myocytes both under resting conditions and during contraction (Knot and Nelson, 1998; Perez et al., 2001).

Both vasodilation and full channel activation occur at LC concentrations well below this steroid CMC, which indicates that these actions are due to the presence of LC monomers in the aqueous phase and not to nonspecific detergent effects on the membrane caused by LC micelles in solution. Lithocholate selectivity on β_1 over β_4 channel subunits also argues for a specific LC-target interaction. Finally, some bile acid analogs that are effective "detergents" (positive curvature-forming lipids) fail to activate myocyte BK channels (Dopico et al., 2002). Collectively, these results strongly support the idea that LC activates BK channels *via* a selective interaction with a steroid target secondary to the presence of LC monomers in solution.

Lithocholate monomers activate the channel independently of cell integrity, cytosolic mediators, or steroid metabolism. Instead, channel activation results from the interaction of LC with the channel protein complex and its immediate lipid microenvironment, the channel β_1-

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

subunit behaving as the "functional target" of LC. Whether channel activation requires LC binding to specific sites in the β_1-subunit itself, or the subunit sensing of LC located somewhere else in the subunit proteolipid vicinity, remains to be determined. However, it is clear from our data that the BK β_1-subunit behaves as the specific LC sensor. Furthermore, this subunit is necessary for LC to dilate the intact artery. The fact that genetic ablation of β_1-subunits prevents LC from dilating small, resistance arteries appears to indicate that the animal fails to develop compensatory mechanisms that could render the myocyte BK channel and the artery sensitive to LC dilation in the absence of β_1-subunits. In brief, we have identified the BK β_1-subunit as the functional target that mediates endothelium-independent LC dilation of intact and pressurized resistance arteries.

In particular, LC-induced dilation of small *cerebral* arteries (~10% increase in diameter) will result in a robust increase in CBF of ~30%, raising the speculation that LC-related analogs could be developed and, eventually, used clinically as cerebrovascular dilators. This possibility acquires particular relevance considering that 1) stroke remains the third leading cause of death and first cause of long-term disability in the US (see www.americanheart.org); 2) >88% of strokes are ischemic (Williams et al., 2003), in which impaired vasomotion may be found; 3) biomedical research has largely failed to provide effective and safe cerebrovascular dilators (Legos et al., 2002); 4) endothelial-mediated vasodilation is impaired in several processes that affect cerebral vessels, such as atherosclerosis and vasospasm. Notably, LC-dilation does not require a functional endothelium (Fig. 1); 5) while several other steroids (17β-estradiol (Valverde et al., 1999), xenoestrogens (Dick and Sanders, 2001; Perez, 2005), androgens (Deenadayalu et al., 2001) and glucocorticoids (King et al., 2006)) activate BK channels, the effects of these agents on cerebrovascular myocyte BK channels and/or tone have not been demonstrated; and 6) these steroids have widespread hormonal actions, which may preclude/limit their clinical use as vasodilators. Thus, pinpointing the LC-myocyte BK β_1-subunit interaction as

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

a mechanism leading to cerebrovascular dilation may be a first step for designing newer and safer steroid-based agents to help in the pharmacological treatment of cerebrovascular ischemic disease.

Lithocholate actions differ in several critical aspects from those of other steroids reported to modulate BK channels. At micromolar (1-30 μM) concentrations, 17β-estradiol increases BK (hslo) channel activity by interacting with the channel β_1-subunit (Valverde et al., 1999). In contrast to LC, 17β-estradiol was also found to be a potent activator of BK channels containing either β_2- or β_4-subunits (King et al., 2006). Furthermore, it has been reported that 17β-estradiol at submicromolar concentrations (0.01-1 μM) can modulate BK activity through an interaction between the steroid and the channel α-subunit (Korovkina et al., 2004). Finally, it has been suggested that 17β-estradiol dilation of coronary arteries *via* BK channels is not the result of a direct action on the channel but mediated through NO/cGMP-mediated pathways (White et al., 2002).

Tamoxifen (a xenoestrogen) and analogs have complex actions on BK activity: increase and decrease in P_o have both been reported, this dual modulation being related to basal P_o before drug application (Dick and Sanders, 2001; Perez, 2005; Duncan, 2005). In contrast, LC increases P_o at all voltages, Ca^{2+}_i, and levels of P_o tested. Furthermore, under some conditions (Perez, 2005; Duncan, 2005), the β_1-subunit is not necessary for tamoxifen to evoke its complex actions on BK channels, the α-subunit being sufficient. Finally, tamoxifen and analogs decrease unitary current amplitude at concentrations as low as 1-10 μM (Duncan, 2005). This action might counterbalance the drug-induced increase in P_o, with consequent reduction in drug potentiation of total BK current and, thus, vasodilation. Instead, the requirement for β_1-subunits to increase P_o and the lack of effects on unitary conductance are observed at all LC concentrations. Thus, in contrast to tamoxifen and analogs, LC modification of BK channel function is limited to that of a gating modifier.

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

Cholesterol at concentrations found in cell membranes reduces BK channel P_o; not only is the final effect opposite to that of LC, but also α-subunits are sufficient for cholesterol action (Bolotina et al., 1989; Crowley et al., 2003). Finally, a recent paper describes that corticosterone activates β_4-containing BK channels more effectively than β_2-containing BK channels, the opposite being true for dehydroepiandrosterone. Testosterone appears not to discriminate among channels containing these two β-subunits (King et al., 2006). In contrast, LC concentrations that are maximally effective in activating cbv1+β_1 channels completely fail to modulate cbv1+β_4 channels.

Dehydrosoyasaponin-1 (DHS-1), a complex molecule that contains a steroidal nucleus, was reported to modulate BK channels through an interaction with the β_1-subunit (Giangiacomo et al., 1998). DHS-1 is effective only when accessing the channel from the cytosolic side of the membrane, limiting its application to tissue/organ studies. In contrast, LC and structural analogs are similarly effective when applied to the external or internal membrane surface (Dopico et al., 2002). DHS-1 action is also strongly voltage-dependent, while LC is not. Finally, it is currently unknown whether other β-subunits (other than β_1) may render BK channels sensitive to nanomolar concentrations of DHS-1. Critically, the fact that LC is not sensed by α+β_4 channel complexes allows us to speculate that LC and its analogs might be used to selectively target tissues/organs that contain high amounts of β_1-subunits (i.e., smooth muscle), as opposed to others rich in α+β_4 complexes [i.e., CNS, in which BK channel activation would affect neuronal excitability (Meredith et al., 2006) and/or neurotransmitter release (Brenner et al., 2005)]. In brief, based on these comparisons with other steroids that modulate BK channels, LC and, likely, its synthetic analogs (Dopico et al., 2002), may represent a unique tool to probe the presence of functional β_1-subunits and/or modulate smooth muscle BK channel activity.

The exact locus of LC action remains speculation. The lateral chain of bile acids contains a carboxylate that is largely ionized at physiological pH (7.35-7.4) at which our experiments were

conducted. The fact that LC action on cbv1+β_1 channel P_o is voltage-independent (suggesting that the ionized carboxylate is not sensed across the voltage field) is consistent with the charged lateral chain residing in or nearby the aqueous solution. The overall hydrophobicity of the steroid nucleus will very likely place it within the lipid bilayer. Remarkably, in contrast to other steroids, LC and analogs are planar amphiphiles. They present a bean-shaped molecule with two clear-cut "planes" or "hemispheres": a concave, polar and a convex, hydrophobic hemisphere. Notably, the planar polarity of the bile acid ring structure is critical for these steroids to increase BK channel P_o (Dopico et al., 2002). Data using chimeric β_1- and β_4-subunits in which transmembrane-cytosolic end and the extracellular loop have been swapped indicate that it is the former region what determines LC sensitivity (Bukiya, Liu, Toro, and Dopico, unpublished). The β_1-subunit transmembrane regions may bring ideal interfaces for LC membrane intercalation, with the hydrophobic hemisphere of the planar amphiphile facing the bilayer lipids and the hemisphere containing the polar hydroxyl facing the β_1-subunit. In this putative model of LC location, however, the presence of polar groups on one side of the bile acid requires some polar surface to diminish the energetic cost of inserting the steroid polar groups within the hydrophobic environment of the bilayer core. Interestingly, the β_1-subunit contains an unusually high number of Thr residues in its transmembrane segments. Furthermore, β_4-subunits, which fail to sense LC, largely lack these polar residues in their transmembrane segments. Systematic mutagenesis combined with molecular modeling will determine which (if any) of the polar residues present in β_1- but absent in β_4-subunits is (are) critical for interacting with bile acids. While the exact locus of LC action on BK channels remains to be determined, it is clear from our study that the channel β_1-subunit behaves as the bile acid sensor.

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgements

Authors deeply thank Robert Brenner (University of Texas at San Antonio) and Richard Aldrich (University of Texas at Austin) for their generous gift of β_1-knockout mice, P. Liu for initially probing LC on cerebrovascular BK channels, Jonathan Jaggar for helpful comments, David Armbruster for critical reading of the manuscript, and M. Asuncion-Chin for technical assistance.

References

Asher C, Wald H, Rossier B, Garty H (1996) Aldosterone-induced increase in the abundance of Na+ channel subunits. *Am J Physiol* 271:C605-611.

Bolotina V, Omelyanenko V, Heyes B, Ryan U, Bregestovski P (1989) Variations of membrane cholesterol alter the kinetics of Ca2(+)-dependent K+ channels and membrane fluidity in vascular smooth muscle cells. *Pflugers Arch* 415:262-268.

Bomzon A, Ljubuncic P (1995) Bile acids as endogenous vasodilators? *Biochem Pharmacol* 49:581-589.

Brenner R, Chen Q, Vilaythong A, Toney G, Noebels J, Aldrich R (2005) BK channel beta4 subunit reduces dentate gyrus excitability and protects against temporal lobe seizures. *Nat Neurosci* 8:1752-1759.

Brenner R, Jegla T, Wickenden A, Liu Y, Aldrich R (2000a) Cloning and characterization of novel large conductance calcium-activated potassium channel β subunits hKCNMB3 and hKCNMB4. *J Biol Chem* 275:6453-6461.

Brenner R, Perez G, Bonev A, Eckman D, Kosek J, Wiler S, Patterson A, Nelson M, Aldrich R (2000b) Vasoregulation by the beta1 subunit of the calcium-activated potassium channel. *Nature* 407:870-876.

Crowley J, Treistman S, Dopico AM (2003) Cholesterol antagonizes ethanol potentiation of human brain BKCa channels reconstituted into phospholipid bilayers. *Mol Pharmacol* 64:365-372.

Deenadayalu VP, White RE, Stallone JN, Gao X, Garcia A (2001) Testosterone relaxes coronary arteries by opening the large-conductance, calcium-activated potassium channel. *Am J Physiol Heart Circ Physiol* 281:H1720-1727.

Dick G, Sanders K (2001) (Xeno)estrogen sensitivity of smooth muscle BK channels conferred by the regulatory β1 subunit. *J Biol Chem* 276:44835-44840.

Dietrich A, Mederos Y Schnitzler M, Gollasch M, Gross V, Storch U, Dubrovska G, Obst M, Yildirim E, Salanova B, Kalwa H, Essin K, Pinkenburg O, Luft FC, Gudermann T, Birnbaumer L (2005) Increased vascular smooth muscle contractility in TRPC6-/- mice. *Mol Cell Biol* 25:6980-6989.

Dopico A, Anantharam V, Treistman S (1998) Ethanol increases the activity of Ca2+-dependent K+ (mslo) channels: Functional interaction with cytosolic Ca2+. *J Pharmacol Exp Ther* 284:258-268.

Dopico A, Walsh J, Singer J (2002) Natural bile acids and synthetic analogues modulate large conductance Ca2+-activated K+ (BKca) channel activity in smooth muscle cells. *J Gen Physiol* 119:251-273.

Duncan R (2005) Tamoxifen alters gating of the BK α subunit and mediates enhanced interactions with the avian β subunit. *Biochem Pharmacol* 70:47-58.

Faraci FM, Sobey CG (1998) Role of potassium channels in regulation of cerebral vascular tone. *J Cereb Blood Flow Metab* 18:1047-1063.

Giangiacomo KM, Kamassah A, Harris G, McManus O (1998) Mechanism of maxi-K channel activation by dehydrosoyasaponin-I. *J Gen Physiol* 112:485-501.

Gourley J, Heistad D (1984) Characteristics of reactive hyperemia in the cerebral circulation. *Am J Physiol* 246:52-58.

Jaggar J, Li A, Parfenova H, Liu J, Umstot E, Dopico A, Leffler C (2005) Heme is a carbon monoxide receptor for large-conductance Ca2+-activated K+ channels. *Circ Res* 97:805-812.

Jaggar J, Porter V, Lederer W, Nelson M (2000) Calcium sparks in smooth muscle. *Am J Physiol Cell Physiol* 278:C235-256.

King JT, Lovell PV, Rishniw M, Kotlikoff M, Zeeman M, McCobb D (2006). Beta2 and beta4 subunits of BK channels confer differential sensitivity to acute modulation by steroid hormones. *J Neurophysiol* 95:2878-2888.

Knot HJ, Nelson M (1998) Regulation of arterial diameter and wall [Ca2+] in cerebral arteries of rat by membrane potential and intravascular pressure. *J Physiol* 508:199-209.

Korovkina VP, Brainard AM, Ismail P, Schmidt T, England S (2004) Estradiol binding to maxi-K channels induces their down-regulation via proteasomal degradation. *J Biol Chem* 279:1217-1223.

Legos J, Tuma R, Barone F (2002) Pharmacological interventions for stroke: failures and future. *Expert Opin Investig Drugs* 11:603-614.

Liu P, Ahmed A, Jaggar J, Dopico A (2004) Essential role for smooth muscle BK channels in alcohol-induced cerebrovascular constriction. *Proc Natl Acad Sci U S A* 101:18217-18222.

Ljubuncic P, Said O, Ehrlich Y, Meddings J, Shaffer E, Bomzon A (2000) On the in vitro vasoactivity of bile acids. *Br J Pharmacol* 131:387-398.

Lovell PV, King J, McCobb DP (2004) Acute modulation of adrenal chromaffin cell BK channel gating and cell excitability by glucocorticoids. *J Neurophysiol* 91:561-570.

Meera P, Wallner M, Jiang Z, Toro L (1996) A calcium switch for the functional coupling between α (hslo) and β subunits (Kv, Caβ) of maxi K channels. *FEBS Lett* 382:84-88.

Meera P, Wallner M, Toro L (2000) A neuronal β subunit (KCNMB4) makes the large conductance, voltage- and Ca2+- activated K+ channels resistant to charybdotoxin and iberiotoxin. *Proc Natl Acad Sci USA* 97:5562-5567.

Meredith A, Wiler S, Miller B, Takahashi J, Fodor A, Ruby N, Aldrich R (2006) BK calcium-activated potassium channels regulate circadian behavioral rhythms and pacemaker output. *Nat Neurosci* 9:1041-1049.

Nimigean CM, Magleby KL (2000) 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. *J Gen Physiol* 115:719-736.

Orio P, Rojas P, Ferreira G, Latorre R (2002) New disguises for an old channel: MaxiK channel beta-subunits. *News Physiol Sci* 17:156-161.

Ostrow J (1993) Metabolism of bile salts in cholestasis in humans, in *Hepatic Transport and Bile Secretion: Physiology and Pathophysiology* (Tavoloni N and Berk P eds) pp 673-712, Raven Press, New York.

Pak JM, Lee SS (1993) Vasoactive effects of bile salts in cirrhotic rats: in vivo and in vitro studies. *Hepatology* 18:1175-1181.

Perez G (2005) Dual effect of tamoxifen on arterial KCa channels does not depend on the presence of the beta1 subunit. *J Biol Chem* 280:21739-21747.

Perez GJ, Bonev AD, Nelson MT (2001) Micromolar Ca(2+) from sparks activates Ca(2+)-sensitive K(+) channels in rat cerebral artery smooth muscle. *Am J Physiol Cell Physiol* 281:C1769-1775.

Roda A, Cerre C, Fini A, Sipahi A, Baraldini M (1995) Experimental evaluation of a model for predicting micellar composition and concentration of monomeric species in bile salt binary mixtures. *J Pharm Sci* 84:593-598.

Thibault N, Ballet F (1993) Effect of bile acids on intracellular calcium in isolated rat hepatocyte couplets. *Bioch Pharm* 45:289-293.

Valverde MA, Rojas P, Amigo J, Cosmelli D, Orio P, Bahamonde MI, Mann GE, Vergara C, Latorre R (1999) Acute activation of Maxi-K channels (hSlo) by estradiol binding to the beta subunit. *Science* 285:1929-1931.

Watson CS, Gametchu B (2003) Proteins of multiple classes may participate in nongenomic steroid actions. *Exp Biol Med (Maywood)* 228:1272-1281.

White R, Han G, Maunz M, Dimitropoulou C, El-Mowafy A, Barlow R, Catravas J, Snead C, Carrier G, Zhu S, Yu X (2002) Endothelium-independent effect of estrogen on Ca(2+)-activated K(+) channels in human coronary artery smooth muscle cells. *Cardiovasc Res* 53:650-661.

Williams CA, Sheppard T, Marrufo M, Galbis-Reig D, Gaskill A (2003). A brief descriptive analysis of stroke features in a population of patients from a large urban hospital in Richmond, Virginia, a city within the 'stroke belt'. *Neuroepidemiology* 22:31-36.

Footnotes

*Supported by NIH grants HL77424 and AA11560 (A.M.D.) and HL54970 (L.T.). J. Liu is an AHA Postdoctoral Fellow.

Reprints requests to: Alex Dopico

The Univ. Tennessee HSC, Dept. Pharmacology

874 Union Ave.

Memphis, TN 38163

Phone: 901-448-3822; Fax: 901-448-1695

Email: adopico@utmem.edu

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

Legends

Figure 1. Lithocholate dilates pressurized arteries *via* activation of BK channels independently of an intact endothelium. A) Rat cerebral arterial diameter trace showing that after artery development of myogenic tone, acute application of 45 μM lithocholate (LC) causes sustained yet fully reversible dilation. LC action is practically abolished by 55 nM iberiotoxin (Ibtx), a selective BK channel blocker (the vasodilatory "rebound" following Ibtx wash is due to increased flow rate; see Materials and Methods). **B)** Diameter trace showing that LC-induced dilation is unaffected by 0.8 mM 4-aminopyridine (4-AP), a blocker of K_V channels other than BK. In A) and B), vertical dotted lines indicate the times at which arterial diameter was determined. **C)** Averaged diameter in response to LC (n=17), Ibtx+LC (n=4), and 4-AP+LC (n=3). LC specific action on diameter is highlighted by displaying data as percentual changes from values obtained in vehicle with (second and third column) or without (first column) K^+ channel blockers. **D)** LC-induced dilation is similar in intact *vs.* endothelium-denuded arteries (n=5). The presence of a functional endothelium was assessed by responses to endothelium-dependent (10 μM acetylcholine [Ach]; n=4) and independent (10 μM sodium nitroprusside [SNP]; n=5) vasodilators. **Different from intact arteries ($p<0.01$).

Figure 2. Lithocholate at submillimolar concentrations activates native BK channels in freshly isolated rat cerebral artery myocytes. A) Single-channel recordings from an I/O patch excised from an arterial myocyte before, during, and after 45 μM lithocholate (LC). Vehicle-containing solution was applied before (Vhcl) and after (Washout) LC-containing solution. Openings are shown as upward deflections; arrows indicate the baseline; Vm=-40mV, free $Ca^{2+}_i \approx 3$ μM. **)** LC action is concentration-dependent: EC_{50}=46±6 μM; $E_{max} \approx 3000$ μM, at which NP_o reaches ~350% of control (n≥3).

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3. Beta$_1$, but not β_4, subunits confer lithocholate sensitivity to BK channels. Records from I/O patches showing that 150 μM LC fail to increase homomeric cbv1 (i.e., rslo1) (**A**) but enhances heteromeric cbv1+β_1 NP_o (**B**) under identical conditions (Vm=-20 mV, free $Ca^{2+}{}_i \approx 10$ μM). **C)** While LC fails to potentiate cbv1 channels even at 300 μM, LC activates cbv1+β_1 channels in a concentration-dependent manner: $E_{max} \approx 300$ μM; $EC_{50}=43.5 \pm 4.7$ μM. These values are almost identical those obtained with native channels in rat cerebral artery myocytes (Fig. 2). **D)** A logit-log plot of LC action on cbv1+β_1 shows data fitted to a sigmoidal function, which renders a slope=1.32 (see main text). To construct this plot, E_{max} was calculated as the mean of NP_o values obtained at 150 and 300 μM LC. **E)** LC at concentrations that maximally activate native BK and cbv1+β_1 channels fails to activate cbv1+β_4 channels. **F)** Averaged ratios of NP_o in the presence ($NP_{o\,LC}$) and absence ($NP_{o\,Vhcl}$) of 150 μM LC for cbv1 (n=6), cbv1+β_1 (n=6), and cbv1+β_4 (n=9) channels expressed in *Xenopus* oocytes. **Different from cbv1+β_1 ($p<0.01$).

Fig. 4. Lithocholate increases BK unitary currents by increasing P_o secondary to a mild increase in mean open time and a marked decrease in mean closed time. A) Current records from an I/O patch containing a single cbv1+β_1 channel expressed in *Xenopus* oocytes in the absence (left) and presence (right) of 150 μM lithocholate (LC). LC increase in P_o (≈320%) is similar to LC increase in NP_o (≈290±45%; Figs. 3C,4B main text), strongly suggesting that LC action occurs without increase in the number of channels (N); V_m set to +20 mV; free $Ca^{2+}{}_i \approx 10$ μM. Arrows on the left of the top traces of each panel indicate the baseline, and channel openings are shown as downward reflections. **B)** cbv1+β_1 channel dwell-times in the absence and presence of 150 μM LC**.** Both open and closed time distributions could be well fitted with a double-exponential function, indicating the existence of at least two open (fast and slow) and two closed (fast and slow) states. The table shows both the average duration of each component (τ) and its contribution to the total time spent in open (closed) states (as percentage in parentheses).

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

LC increase in P_o (~320%) is caused by a mild increase in the average duration of both short and long open events and a sharp decrease (-60%) in mean close time, the latter basically due to LC-induced destabilization of channel long closures.

Fig. 5. Lithocholate activates BK channels within the physiological ranges of $[Ca^{2+}]_i$ and membrane potential. A) NP_o during exposure of intracellular side of I/O patches to 150 μM LC ($NP_{o\ LC}$) *vs.* NP_o in vehicle-containing solution ($NP_{o\ Vhcl}$) plotted as a function of free $[Ca^{2+}]_i$. Channel NP_o was obtained following coexpression of cbv1 and β_1-subunits in *Xenopus* oocytes. The membrane voltage was set within the rage ±20 mV, and the bath solution contained 0.1 (n=4), 0.3 (n=3), 1 (n=3), 3 (n=7), 10 (n=6), or 30 μM (n=4) free $[Ca^{2+}]_i$. Each column represents the mean±SEM. **B)** Voltage needed for half-maximal increase in BK channel NP_o ($V_{0.5}$) as a function of $[Ca^{2+}]_i$ in the vehicle-containing solution (Vhcl) and in the presence of 150 μM LC. $V_{0.5}$ values were obtained from G/G_{max} curves for I/O macropatches at 0.3, 3, and 10 μM $Ca^{2+}{}_i$. Voltage steps 200 msec duration were applied from -150 to +150 mV with 10-mV increments, $V_{holding}$=0 mV. Each data point represents the mean value±SEM from ≥4 patches (oocytes). At every $[Ca^{2+}]_i$ LC causes a similar leftward shift in $V_{0.5}$ of ~17.7 mV.

Fig. 6. Lithocholate fails to dilate pressurized arteries from β_1-subunit knockout mice. **A)** Arterial diameter traces show that acute 45 μM LC and 55 nM Ibtx cause sustained diameter increase and decrease, respectively, in arteries from *wt* mice (grey trace) but not in arteries from BK β_1-knockout mice (black trace). The small and transient dilation caused by vehicle (Vhcl) is similar in both mice. **B)** Averaged diameter data in response to LC (left) and Ibtx (right) in *wt* (hollow) (n=7) and β_1-knockout (black bars) (n=5) mice. *Different from *wt* mice ($p<0.05$); **Different from *wt* mice ($p<0.01$).

Molecular Pharmacology Fast Forward. Published on April 27, 2007 as DOI: 10.1124/mol.107.034330
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. Characteristics of lithocholic acid- *vs.* vehicle-induced vasodilation.

Parameter	Vehicle (0.1% DMSO)	Lithocholic acid (45 µM)
Rise time (sec)	244.2±16.1	231.9±21.9
Maximal effect (% from diameter before compound application)	110.8±1.6	116.58±1.9 **
Time for full recovery (sec)	347.4±47.1	981.4±129.8 **
Integral (area under the curve) from the time of drug application until complete washout of effect	10,262 ±2,574	18,369± 3,964 **
Effect remaining after ~6 min of washout (percentual increase from diameter before agent application)	None	109.9±1.7 **

**Significantly different from control, vehicle-containing solution ($p<0.01$) (paired Student's *t*-test).

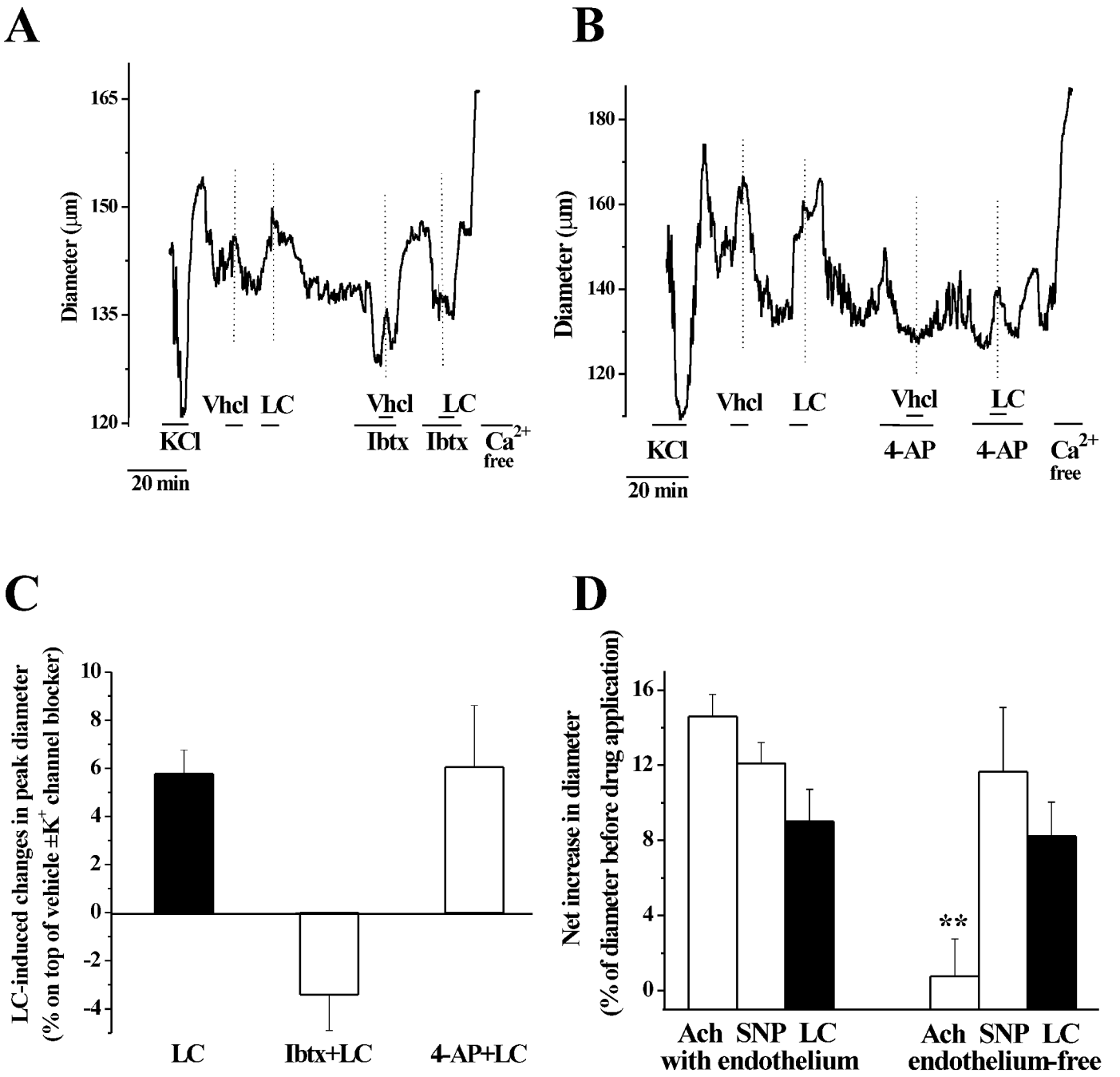

A
Diameter (μm)
165
150
135
120
Vhcl
LC
Vhcl
LC
KCl
Ibtx
Ibtx
Ca2+ free
20 min
B
Diameter (μm)
180
160
140
120
Vhcl
LC
Vhcl
LC
KCl
4-AP
4-AP
Ca2+ free
20 min
C
LC-induced changes in peak diameter (% on top of vehicle ±K+ channel blocker)
10
8
6
4
2
0
-2
-4
LC
Ibtx+LC
4-AP+LC
D
Net increase in diameter (% of diameter before drug application)
16
12
8
4
0
**
Ach SNP LC
with endothelium
Ach SNP LC
endothelium-free

Figure 1

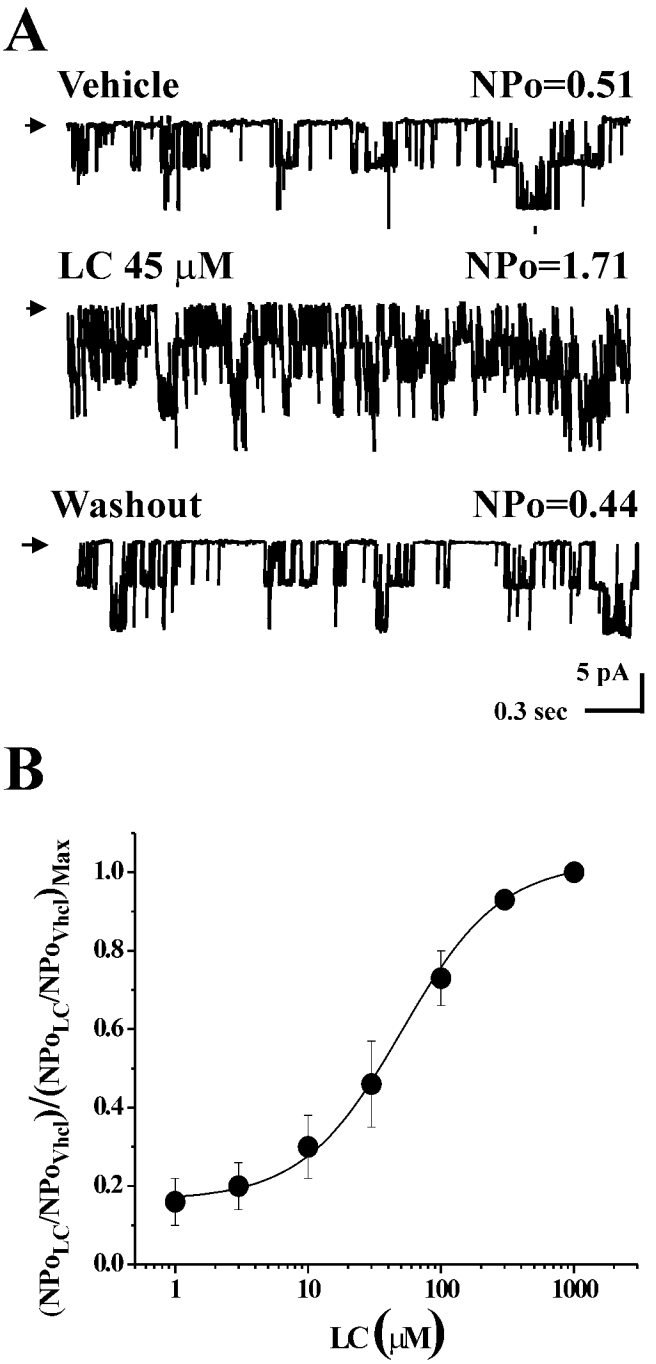

A
Vehicle
NPo=0.51
LC 45 μM
NPo=1.71
Washout
NPo=0.44
5 pA
0.3 sec
B
(NPo_LC/NPo_Vhcl)/(NPo_LC/NPo_Vhcl)_Max
1.0
0.8
0.6
0.4
0.2
0.0
1
10
100
1000
LC (μM)

Figure 2

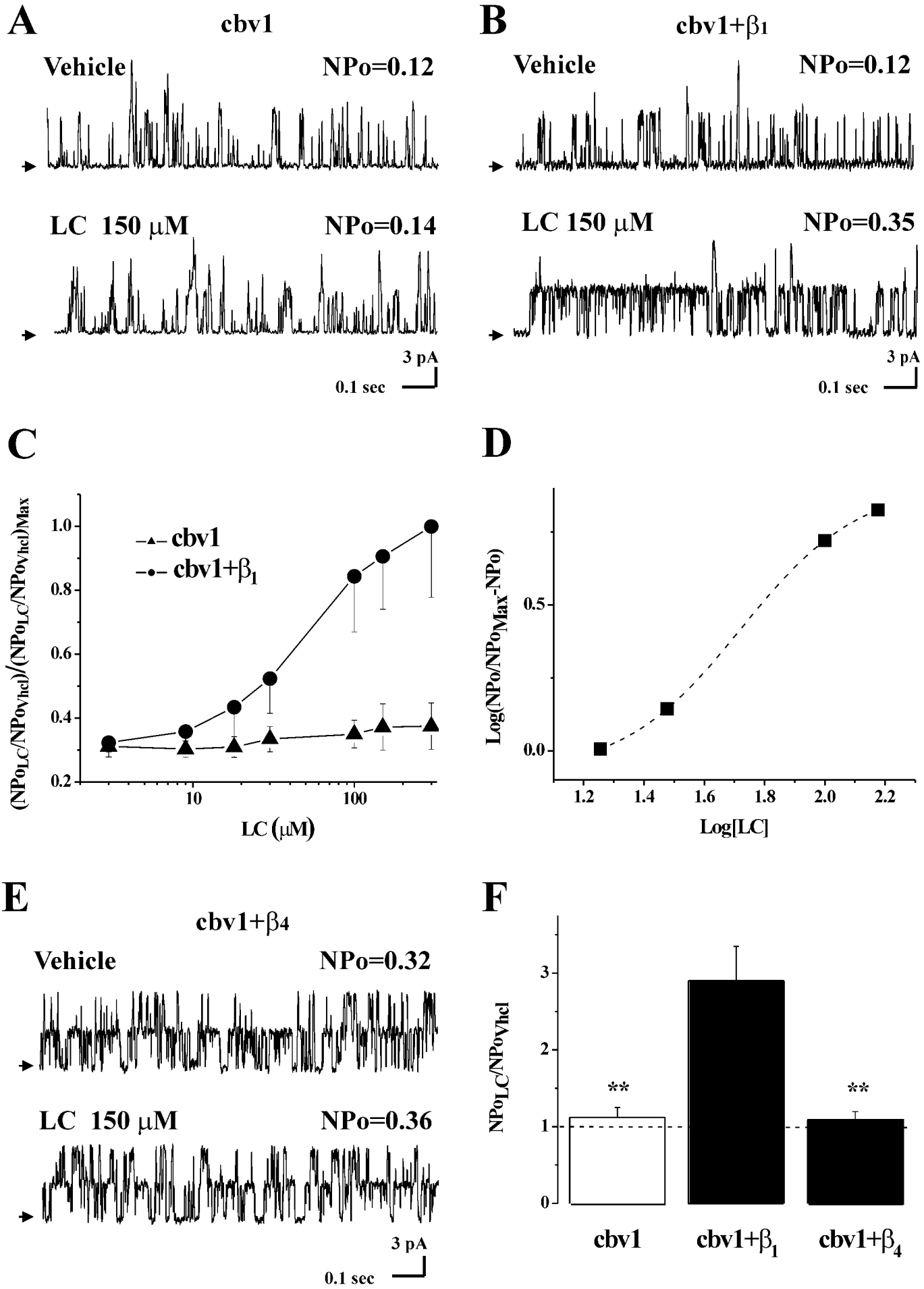
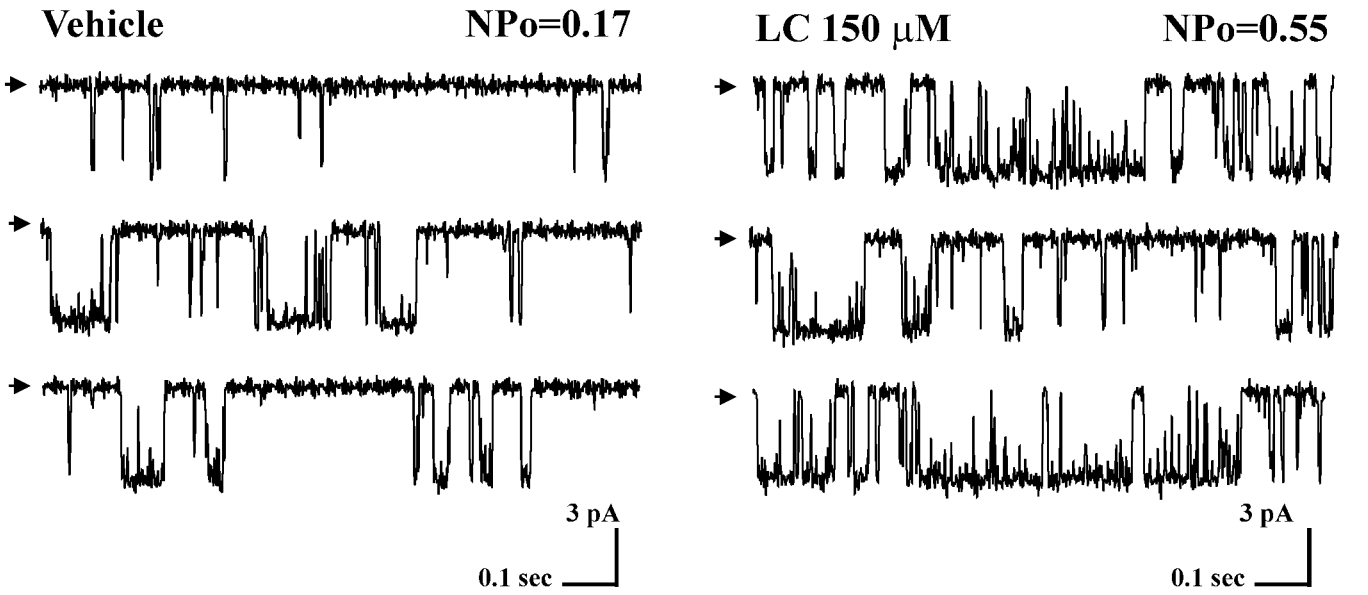

A
cbv1
Vehicle
NPo=0.12
LC 150 μM
NPo=0.14
3 pA
0.1 sec
B
cbv1+β1
Vehicle
NPo=0.12
LC 150 μM
NPo=0.35
3 pA
0.1 sec
C
cbv1
cbv1+β1
(NPoLC/NPoVhcl)/(NPoLC/NPoVhcl)Max
LC (μM)
D
Log(NPo/NPoMax-NPo)
Log[LC]
E
cbv1+β4
Vehicle
NPo=0.32
LC 150 μM
NPo=0.36
3 pA
0.1 sec
F
NPoLC/NPoVhcl
cbv1
cbv1+β1
cbv1+β4

Figure 3

A

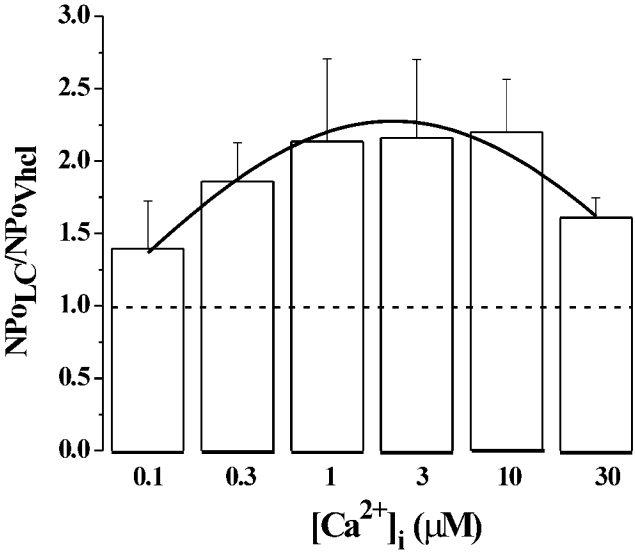


B

	Vehicle (Po=0.17)	LC 150 μM (Po=0.55)
τ open, msec (% of total distribution)	τ fast 1.69±0.013 (51%)	τ fast 2.28±0.37 (43%)
	τ slow 14.97±2.19 (49%)	τ slow 18.25±1.89 (57%)
Mean open time	**8.14±1.14 msec**	**11.32±1.24 msec**
τ close, msec (% of total distribution)	τ fast 1.66±0.24 (56%)	τ fast 1.45±0.16 (66%)
	τ slow 74.57±10.10 (44%)	τ slow 38.00±6.18 (34%)
Mean close time	**33.74±4.57 msec**	**14.06±2.21 msec**

Figure 4

A


3.0
2.5
2.0
1.5
1.0
0.5
0.0
NPo_LC/NPo_Vhcl
0.1
0.3
1
3
10
30
[Ca2+]i (μM)

B

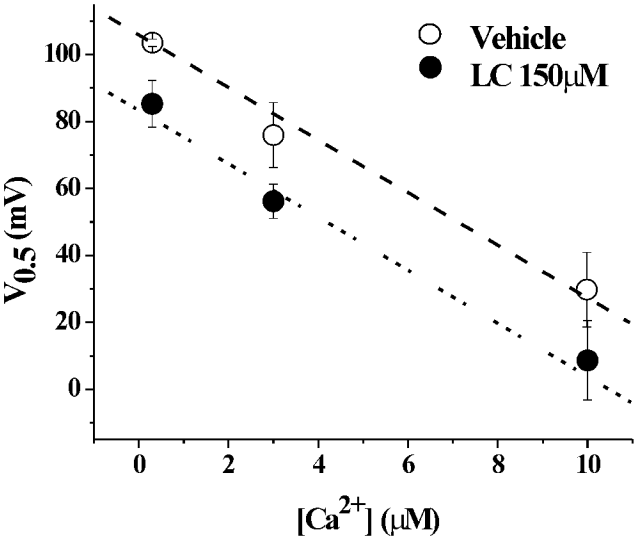

Vehicle
LC 150μM
100
80
60
40
20
0
V0.5 (mV)
0
2
4
6
8
10
[Ca2+] (μM)

Figure 5

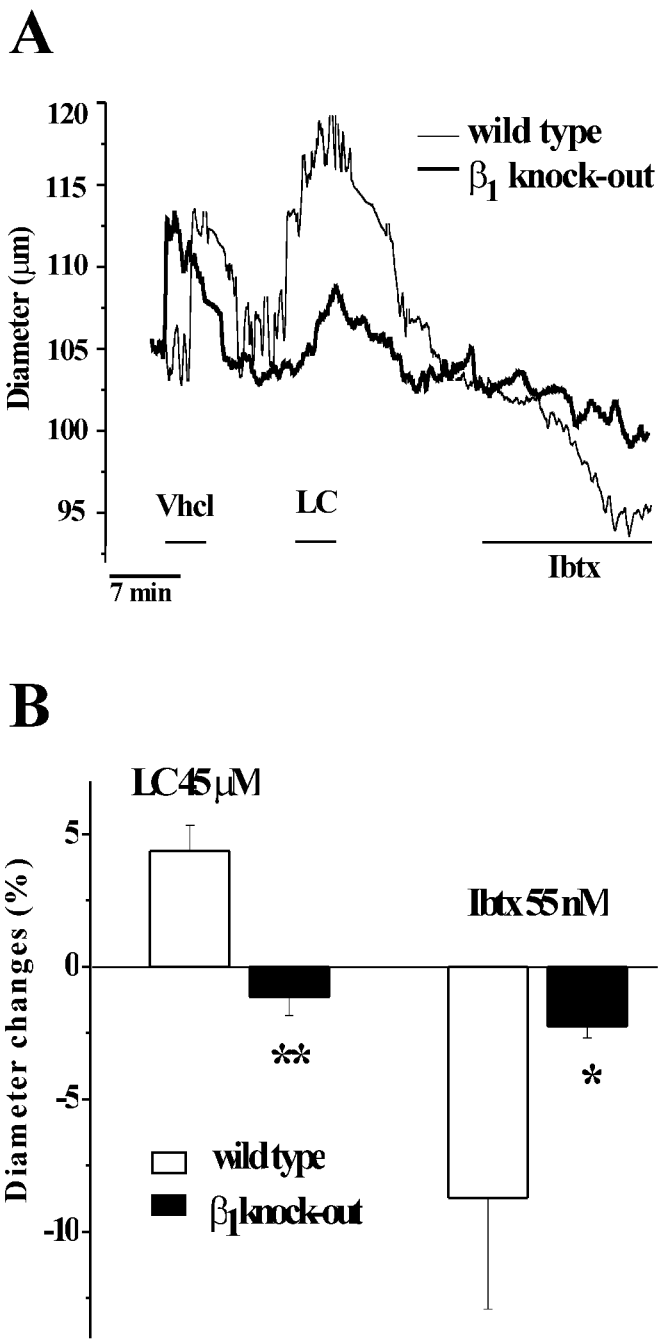
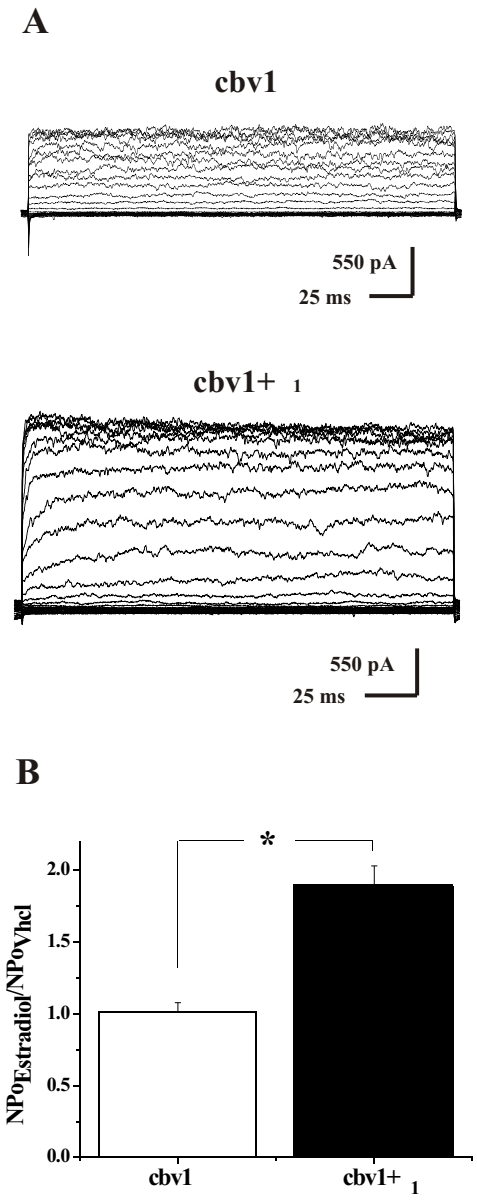
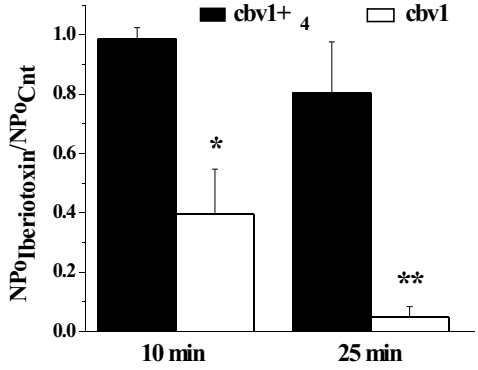

A
120
115
110
105
100
95
Diameter (μm)
wild type
β1 knock-out
Vhcl
LC
Ibtx
7 min
B
LC45μM
Ibtx55nM
5
0
-5
-10
Diameter changes (%)
**
*
wild type
β1knock-out

Figure 6

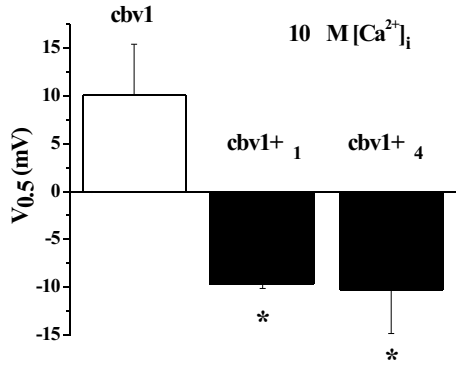


Supplementary Figure 1

A



B



Supplementary Figure 2