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**$\beta_1$  (*KCNMB1*)-subunits mediate lithocholate activation of BK channels and dilation in  
small, resistance arteries**

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ABBREVIATIONS: LC, lithocholate; BK, large-conductance, Ca<sup>2+</sup>-activated K<sup>+</sup>; 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<sub>o</sub>, open channel probability; DMSO, dimethyl sulfoxide; CMC, critical micellar concentration; CBF, cerebral blood flow; K<sub>v</sub>, voltage-gated K<sup>+</sup>; 4-AP, 4-aminopyridine; z, effective valence; DHS-1, dehydrosoyasaponin-1

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## 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  $\mu\text{M}$ ) reversibly increases the diameter of pressurized resistance cerebral arteries by  $\sim 10\%$ , which would result in  $\sim 30\%$  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  $\text{Ca}^{2+}$  concentrations reached in the myocyte whether at rest or during contraction. Channel accessory  $\beta_1$ -subunits, which are predominant in smooth muscle, are necessary for LC to modify channel activity. In contrast,  $\beta_4$ -subunits, which are predominant in neuronal tissues, fail to evoke LC sensitivity. LC activation of  $\text{cbv1}+\beta_1$  and native BK channels display identical characteristics, including  $\text{EC}_{50}$  (46  $\mu\text{M}$ ) and  $\text{E}_{\text{max}}$  ( $\approx 300$   $\mu\text{M}$ ), strongly suggesting that the  $\text{cbv1}+\beta_1$  complex is necessary and sufficient to evoke LC action. Finally, intact arteries from  $\beta_1$ -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  $\beta_1$ -subunit as the molecule that senses LC, which results in myocyte BK channel activation and, thus, endothelial-independent relaxation of small, resistance arteries.

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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  $\mu\text{M}$  (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

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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,  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  (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  $\alpha$  (*KCNMA1*)- and regulatory  $\beta_1$  (*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  $\alpha$ -subunits (termed “cbv1”; AY330293) from myocytes freshly isolated from rat resistance cerebral arteries, we used recombinant BK channels to demonstrate that the channel  $\beta_1$ -subunit acts as the LC sensor. In contrast,  $\beta_4$ -subunits fail to render BK channels sensitive to LC. Moreover, pharmacological block of BK (but not other  $\text{K}_V$  channels that also control myocyte tone) or genetic ablation of BK  $\beta_1$ -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 ( $\approx 250$  g) or 8- to 12-week-old  $\beta_1$ -knockout and C57BL/6 control mice. Rats

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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, Tokyo, Japan) and placed into ice-cold “dissociation medium” (DM) (mM): 0.16 CaCl<sub>2</sub>, 0.49 EDTA, 10 HEPES, 5 KCl, 0.5 KH<sub>2</sub>PO<sub>4</sub>, 2 MgCl<sub>2</sub>, 110 NaCl, 0.5 NaH<sub>2</sub>PO<sub>4</sub>, 10 NaHCO<sub>3</sub>, 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 °C in a polypropylene centrifuge tube, and then incubated in a shaking water bath at 37 °C and 60 oscillations/min. for 15 min. The preparation was then centrifuged several

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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 ( $\geq 5$  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<sub>1</sub>-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<sub>4</sub>-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  $\beta_1$ -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 ( $\beta_1$  or  $\beta_4$ ) ng/ $\mu$ l; 1- $\mu$ l aliquots were stored at -70°C. Cbv1 cRNA was injected alone (2.5 ng/ $\mu$ l) or coinjected with either  $\beta_1$  or  $\beta_4$  (7.5 ng/ $\mu$ l) cRNAs, giving molar ratios  $\geq 6:1$  ( $\beta:\alpha$ ). cRNA injection (23 nl/oocyte)

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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<sup>+</sup> gluconate, 5 EGTA, 1 MgCl<sub>2</sub>, 15 HEPES, 10 glucose, pH 7.35. For experiments with myocytes, KCl substituted for K<sup>+</sup> gluconate. In all experiments, free Ca<sup>2+</sup> in solution was adjusted to the desired value by adding CaCl<sub>2</sub>. In most studies, free Ca<sup>2+</sup> in electrode solution=10 μM. In O/O studies with 17β-estradiol, however, free Ca<sup>2+</sup> in the electrode solution=0.3 μM. Nominal free Ca<sup>2+</sup> was calculated with MaxChelator Sliders (C. Patton, Stanford University, CA) and validated experimentally using Ca<sup>2+</sup>-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<sup>-</sup> 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



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pCLAMP 8.0 (Molecular Devices, Union City, CA). For macropatch recordings, G/G<sub>max</sub>-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<sub>max</sub> 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<sub>o</sub>). NP<sub>o</sub> 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.

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## Results

**Lithocholate Dilates Small, Resistance Arteries *via* BK Channels.** After myogenic tone development at 60 mm Hg, intact arteries reached a diameter of  $154.4 \pm 5.2 \mu\text{m}$  ( $n=17$ ). Maximal contraction and dilation were checked by perfusing the vessel with 60 mM KCl at the beginning, and  $\text{Ca}^{2+}$ -free solution at the end of each experiment (Fig. 1A,B). In all cases ( $n=17$ ), application of 45  $\mu\text{M}$  LC, that is, concentrations well below LC's critical micellar concentration (CMC) ( $\geq 1$  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 ( $\approx 2.8$  times longer); for example, by the time the vehicle effect had totally vanished, LC-induced dilation still represented  $109.9 \pm 1.7\%$  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:  $18,369 \pm 3,964$  *vs.*  $10,262 \pm 2,574$  ( $p < 0.01$ ) (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 ( $\sim 30\%$ ) in cerebral blood flow (CBF), because changes in artery diameter are related to changes in CBF by a factor of  $\sim 3$  (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 ( $-11.8 \pm 3.4\%$ ) ( $n=4$ ) (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

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increase in cytosolic  $\text{Ca}^{2+}$  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  $\text{K}^+$  channels ( $\text{K}_V$ ) 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  $\text{K}_V$  but not BK channels in rat cerebral arteries (Liu et al., 2004). Applying 0.8 mM 4-AP caused an immediate decrease in diameter ( $-9.7 \pm 2.3\%$ ,  $n=4$ ) (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  $\text{K}_V$  blocker ( $+6\%$  over pre-LC values; Fig. 1C). While we cannot rule out some contribution of  $\text{K}_V$  channels other than BK to LC dilation, our results indicate that  $\text{K}_V$  channels other than BK do not play a major role in LC dilation of pressurized small, resistance cerebral arteries. Furthermore, 45  $\mu\text{M}$  LC on top of 4-AP almost totally reverted the vasoconstriction caused by the  $\text{K}_V$  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 ( $n=5$ ).

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The presence of a functional endothelium was assessed by vascular responses to endothelium-dependent (acetylcholine; 10  $\mu$ M) and independent (sodium nitroprusside; 10  $\mu$ M) 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  $\beta_1$ -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  $\mu$ M) 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  $\mu$ M) solution reversibly increased  $NP_o$  in a concentration-dependent fashion:  $EC_{50}=46\pm 6$   $\mu$ M,  $E_{max}\sim 300$   $\mu$ M (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

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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+ $\beta_1$  channels under identical conditions (to evoke measurable levels of  $P_o$  within a sec-min time frame in the absence of  $\beta_1$ -subunits, these studies were conducted at  $Ca^{2+}_i=10 \mu M$ , 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  $\beta_1$ -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  $\sim 20$  mV towards negative potentials) and channel activation by bath application of  $10 \mu M$   $17\beta$ -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  $150 \mu M$  (on top of vehicle) to the internal side of I/O patches failed to activate homomeric cbv1 channels, with average  $NP_o$  reaching  $112 \pm 13\%$  of control ( $p > 0.05$ ;  $n=4$ ) (Figs. 3A,C). Thus, LC activation of cerebrovascular BK channels requires the presence of  $\beta_1$ -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 ( $3-300 \mu M$ ) caused a reversible and concentration-dependent increase in  $NP_o$  of heteromeric cbv1+ $\beta_1$  channels (Figs. 3B,C), with  $EC_{50}=43.5 \pm 4.7 \mu M$  and  $E_{max} \sim 300 \mu M$ , at which  $NP_o$  reached  $290 \pm 45\%$  of control. These values are practically identical to those of native BK channels (see above), indicating that differences in composition/organization between rat cerebrovascular

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myocyte and *Xenopus* oocyte membranes are not critical in LC action on BK channels. The identical LC responses of native cerebrovascular BK and  $cbv1+\beta_1$  channels appear to indicate the involvement of a common target(s) mediating LC action in these two systems, possibly the  $\beta_1$ -subunit itself.

A Hill-like plot for LC-activation of  $cbv1+\beta_1$  channels renders a slope (*apparent* Hill coefficient) of 1.3 (Fig. 3D), which suggests the involvement of at least two “sites” in the  $cbv1+\beta_1$  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  $\beta_1$ -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,

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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:  $228.6 \pm 3.7$  vs.  $234.0 \pm 4.6$  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  $\beta$ -subunit type ( $\beta_1$ ) that is predominant in smooth muscle or could be mediated by other channel accessory-subunits, we tested LC action on  $cbv1+\beta_4$  channels. When coexpressed with  $\alpha$ -subunits,  $\beta_4$ -subunits introduce a hyperpolarizing shift in  $V_{0.5}$  similar to that caused by  $\alpha+\beta_1$  coexpression. In addition,  $\beta_4$ -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  $cbv1+\beta_1$  channels,  $cbv1+\beta_4$  channels were consistently refractory to LC action (8/8 patches), even when tested at concentrations (150  $\mu$ M) that were close to  $E_{max}$  in both  $cbv1+\beta_1$  and native BK channels (Fig. 3E,F);  $cbv1+\beta_4$   $NP_o$  reached  $109 \pm 11\%$  of control (NS, also not significantly different from LC action on  $cbv1$  homomeric channels). Therefore,  $\beta_1$ - but not  $\beta_4$ -subunits confer LC sensitivity to cerebrovascular BK channels.

**Lithocholate Effectively Activates BK Channels within Physiological Ranges of  $Ca^{2+}_i$  and Membrane Voltage.**  $\beta_1$ -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; Nimigeon 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  $cbv1+\beta_1$  channel  $P_o$  by using a wide voltage range ( $\pm 150$  mV) and  $Ca^{2+}_i$  levels that expanded those in the myocyte under physiological conditions (0.15-0.3  $\mu$ M at rest; up to 10-30  $\mu$ M in the vicinity of BK

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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<sub>o</sub> was unnoticeable when recorded in solutions having zero Ca<sup>2+</sup> 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<sub>1</sub>-mediated mechanism, as at “zero” or subactivatory Ca<sup>2+</sup><sub>i</sub>, β<sub>1</sub>-subunit modification of gating does not translate into an evident change in overall P<sub>o</sub> (Nimigean and Magleby, 2000). Furthermore, LC-activation (as a percentage of NP<sub>o</sub> in vehicle) increased with Ca<sup>2+</sup><sub>i</sub>: from 139.9±32.9 (n=4; p<0.05) at 0.1 μM Ca<sup>2+</sup> to a maximal effect of 244.1±58.9% (n=3; p<0.01) at 1 μM Ca<sup>2+</sup>. This maximum remained steady within the 1 to 10 μM Ca<sup>2+</sup><sub>i</sub> range (n=16), to decrease with higher Ca<sup>2+</sup><sub>i</sub> (e.g., at 30 μM, NP<sub>o</sub> 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<sup>2+</sup> range that spans from resting levels to those reached during myocyte contraction. Remarkably, LC activation is most effective at Ca<sup>2+</sup> levels reached near the BK channel during cerebral artery myocyte contraction (Perez et al., 2001).

Next, we evaluated LC action on cbv1+β<sub>1</sub>-mediated currents as a function of applied voltage, exposing I/O macropatches to Ca<sup>2+</sup><sub>i</sub> at which LC activation of BK channels is robust: 0.3, 3, and 10 μM Ca<sup>2+</sup><sub>i</sub>. From G/G<sub>max</sub> vs. V<sub>m</sub> plots fitted to a Boltzmann relationship, we obtained V<sub>0.5</sub>=101.9±1.2 (n=3), 74±10 (n=3), and 32.3±11.2 mV (n=5), respectively. At every Ca<sup>2+</sup><sub>i</sub> tested, LC (150 μM) shifted the V<sub>0.5</sub> by ≈-17.7 mV (Fig. 5B) without changing the slope of the plot. Thus, at any constant Ca<sup>2+</sup><sub>i</sub>, 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<sup>2+</sup><sub>i</sub>: 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<sub>1</sub>-mediated action on channel gating (Brenner et al., 2000a).



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Together, the data show that LC is an effective activator of BK channels *via* their  $\beta_1$ -subunits at physiologically relevant  $Ca^{2+}_i$  and voltages.

**Lithocholate Fails to Induce Cerebrovascular Dilation in  $\beta_1$ -Knockout Mice.** To determine the impact of LC targeting of BK  $\beta_1$ -subunits on organ function, we evaluated LC action on the arterial diameter of pressurized cerebral arteries from  $\beta_1$ -knockout *vs.* *wt* C57BL/6 mice (controls). To verify the presence of functional  $\beta_1$ -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  $\beta_1$ -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  $\beta_1$ -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  $\mu$ M 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  $\beta_1$ -knockout mice ( $n=5$ ) (Fig. 6A,B), indicating that in intact cerebral arteries the presence of BK  $\beta_1$ -subunits is crucial for LC dilation.

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## 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  $\beta_1$ -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  $\beta_1$  over  $\beta_4$  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  $\beta_1$ -

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subunit behaving as the “functional target” of LC. Whether channel activation requires LC binding to specific sites in the  $\beta_1$ -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  $\beta_1$ -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  $\beta_1$ -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  $\beta_1$ -subunits. In brief, we have identified the BK  $\beta_1$ -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](http://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 (17 $\beta$ -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  $\beta_1$ -subunit interaction as

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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  $\mu\text{M}$ ) concentrations,  $17\beta$ -estradiol increases BK (hslo) channel activity by interacting with the channel  $\beta_1$ -subunit (Valverde et al., 1999). In contrast to LC,  $17\beta$ -estradiol was also found to be a potent activator of BK channels containing either  $\beta_2$ - or  $\beta_4$ -subunits (King et al., 2006). Furthermore, it has been reported that  $17\beta$ -estradiol at submicromolar concentrations (0.01-1  $\mu\text{M}$ ) can modulate BK activity through an interaction between the steroid and the channel  $\alpha$ -subunit (Korovkina et al., 2004). Finally, it has been suggested that  $17\beta$ -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,  $\text{Ca}^{2+}_i$ , and levels of  $P_o$  tested. Furthermore, under some conditions (Perez, 2005; Duncan, 2005), the  $\beta_1$ -subunit is not necessary for tamoxifen to evoke its complex actions on BK channels, the  $\alpha$ -subunit being sufficient. Finally, tamoxifen and analogs decrease unitary current amplitude at concentrations as low as 1-10  $\mu\text{M}$  (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  $\beta_1$ -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.

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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  $\alpha$ -subunits are sufficient for cholesterol action (Bolotina et al., 1989; Crowley et al., 2003). Finally, a recent paper describes that corticosterone activates  $\beta_4$ -containing BK channels more effectively than  $\beta_2$ -containing BK channels, the opposite being true for dehydroepiandrosterone. Testosterone appears not to discriminate among channels containing these two  $\beta$ -subunits (King et al., 2006). In contrast, LC concentrations that are maximally effective in activating  $\alpha\beta_1$  channels completely fail to modulate  $\alpha\beta_4$  channels.

Dehydrosoyasaponin-1 (DHS-1), a complex molecule that contains a steroidal nucleus, was reported to modulate BK channels through an interaction with the  $\beta_1$ -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  $\beta$ -subunits (other than  $\beta_1$ ) may render BK channels sensitive to nanomolar concentrations of DHS-1. Critically, the fact that LC is not sensed by  $\alpha\beta_4$  channel complexes allows us to speculate that LC and its analogs might be used to selectively target tissues/organs that contain high amounts of  $\beta_1$ -subunits (i.e., smooth muscle), as opposed to others rich in  $\alpha\beta_4$  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  $\beta_1$ -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

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conducted. The fact that LC action on  $\text{cbv1}+\beta_1$  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  $\beta_1$ - and  $\beta_4$ -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  $\beta_1$ -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  $\beta_1$ -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  $\beta_1$ -subunit contains an unusually high number of Thr residues in its transmembrane segments. Furthermore,  $\beta_4$ -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  $\beta_1$ - but absent in  $\beta_4$ -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  $\beta_1$ -subunit behaves as the bile acid sensor.

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**Footnotes**

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## 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  $\mu$ M 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  $\mu$ M acetylcholine [Ach]; n=4) and independent (10  $\mu$ M 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  $\mu$ M 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;  $V_m = -40$ mV, free  $Ca^{2+}_i \approx 3$   $\mu$ M. **)** LC action is concentration-dependent:  $EC_{50} = 46 \pm 6$   $\mu$ M;  $E_{max} \approx 3000$   $\mu$ M, at which  $NP_o$  reaches  $\sim 350\%$  of control (n $\geq 3$ ).

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**Figure 3. Beta<sub>1</sub>, but not β<sub>4</sub>, 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<sub>1</sub> NP<sub>o</sub> (B) under identical conditions (V<sub>m</sub>=-20 mV, free Ca<sup>2+</sup><sub>i</sub>≈10 μM). C) While LC fails to potentiate cbv1 channels even at 300 μM, LC activates cbv1+β<sub>1</sub> channels in a concentration-dependent manner: E<sub>max</sub>≈300 μM; EC<sub>50</sub>=43.5±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<sub>1</sub> shows data fitted to a sigmoidal function, which renders a slope=1.32 (see main text). To construct this plot, E<sub>max</sub> was calculated as the mean of NP<sub>o</sub> values obtained at 150 and 300 μM LC. E) LC at concentrations that maximally activate native BK and cbv1+β<sub>1</sub> channels fails to activate cbv1+β<sub>4</sub> channels. F) Averaged ratios of NP<sub>o</sub> in the presence (NP<sub>o LC</sub>) and absence (NP<sub>o vhel</sub>) of 150 μM LC for cbv1 (n=6), cbv1+β<sub>1</sub> (n=6), and cbv1+β<sub>4</sub> (n=9) channels expressed in *Xenopus* oocytes. \*\*Different from cbv1+β<sub>1</sub> (p<0.01).

**Fig. 4. Lithocholate increases BK unitary currents by increasing P<sub>o</sub> 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<sub>1</sub> channel expressed in *Xenopus* oocytes in the absence (left) and presence (right) of 150 μM lithocholate (LC). LC increase in P<sub>o</sub> (≈320%) is similar to LC increase in NP<sub>o</sub> (≈290±45%; Figs. 3C,4B main text), strongly suggesting that LC action occurs without increase in the number of channels (N); V<sub>m</sub> set to +20 mV; free Ca<sup>2+</sup><sub>i</sub>≈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<sub>1</sub> 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).

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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  $\mu$ M LC ( $NP_{oLC}$ ) vs.  $NP_o$  in vehicle-containing solution ( $NP_{oVhcl}$ ) plotted as a function of free  $[Ca^{2+}]_i$ . Channel  $NP_o$  was obtained following coexpression of *cbv1* and  $\beta_1$ -subunits in *Xenopus* oocytes. The membrane voltage was set within the range  $\pm 20$  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  $\mu$ M (n=4) free  $[Ca^{2+}]_i$ . Each column represents the mean  $\pm$  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  $\mu$ M LC.  $V_{0.5}$  values were obtained from  $G/G_{max}$  curves for I/O macropatches at 0.3, 3, and 10  $\mu$ M  $Ca^{2+}_i$ . 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  $\pm$  SEM from  $\geq 4$  patches (oocytes). At every  $[Ca^{2+}]_i$  LC causes a similar leftward shift in  $V_{0.5}$  of  $\sim 17.7$  mV.

**Fig. 6. Lithocholate fails to dilate pressurized arteries from  $\beta_1$ -subunit knockout mice.**

**A)** Arterial diameter traces show that acute 45  $\mu$ M 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  $\beta_1$ -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  $\beta_1$ -knockout (black bars) (n=5) mice. \*Different from *wt* mice (p<0.05); \*\*Different from *wt* mice (p<0.01).

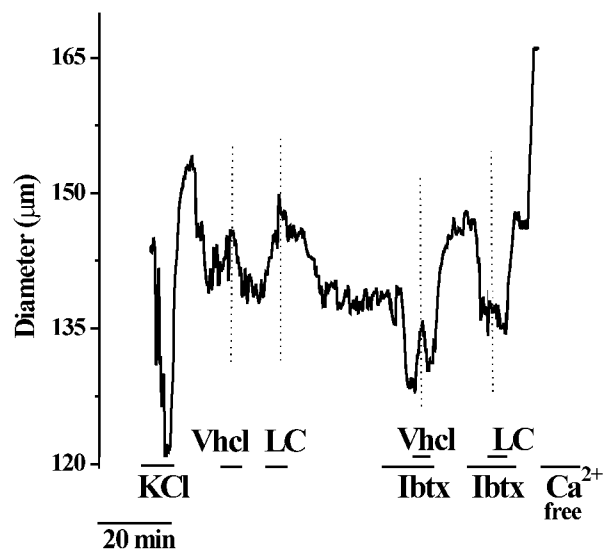
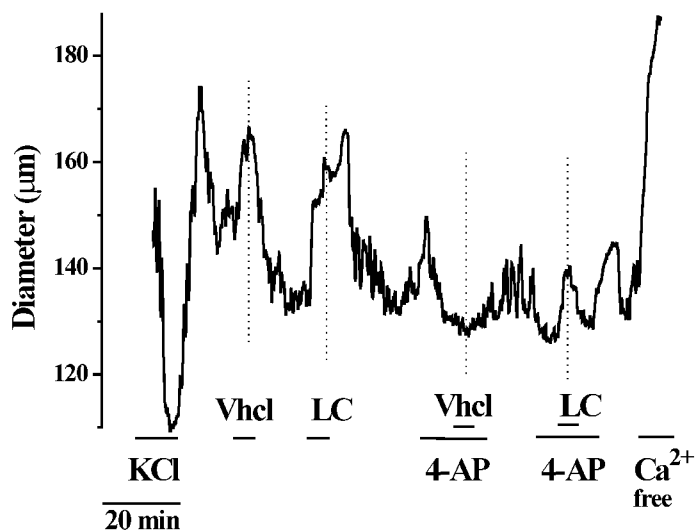
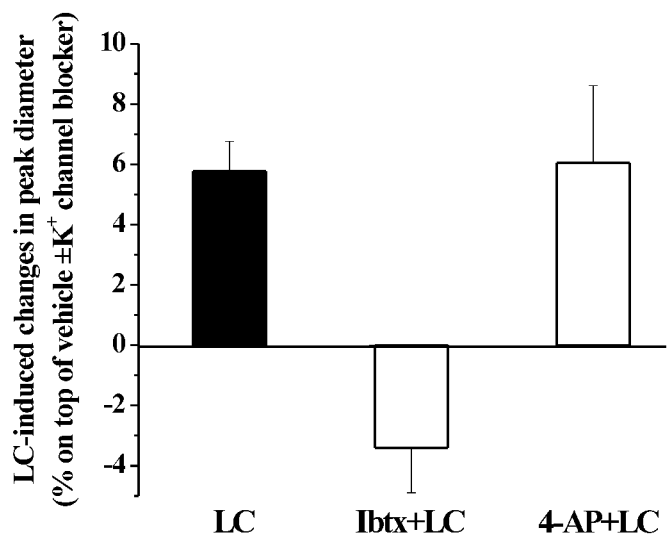
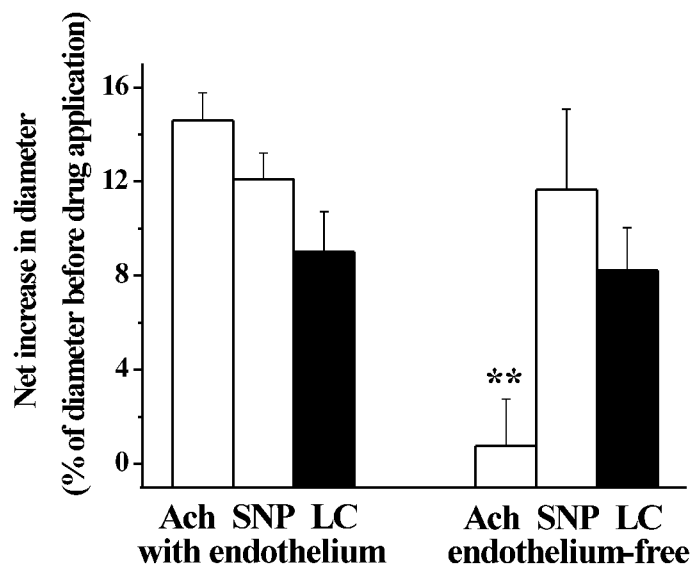


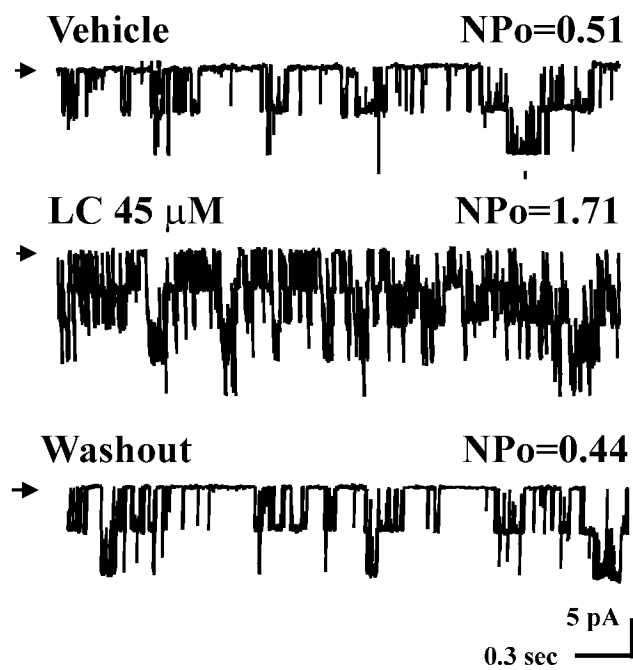
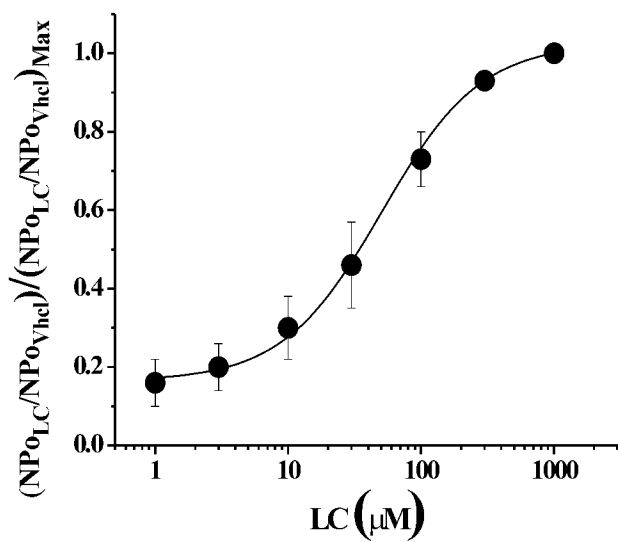
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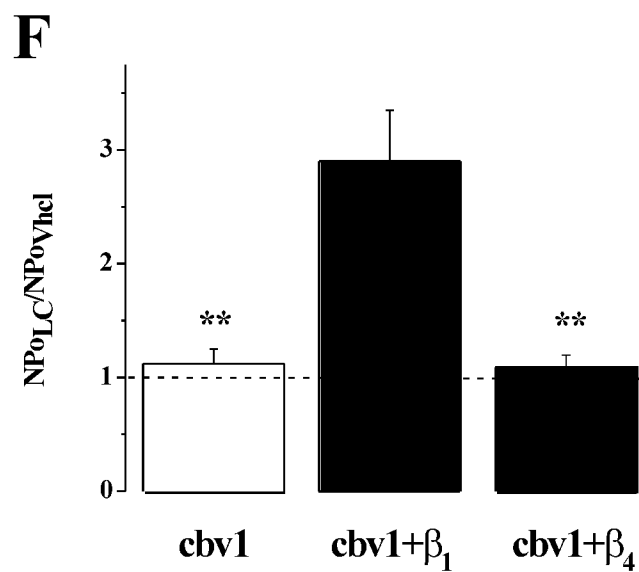
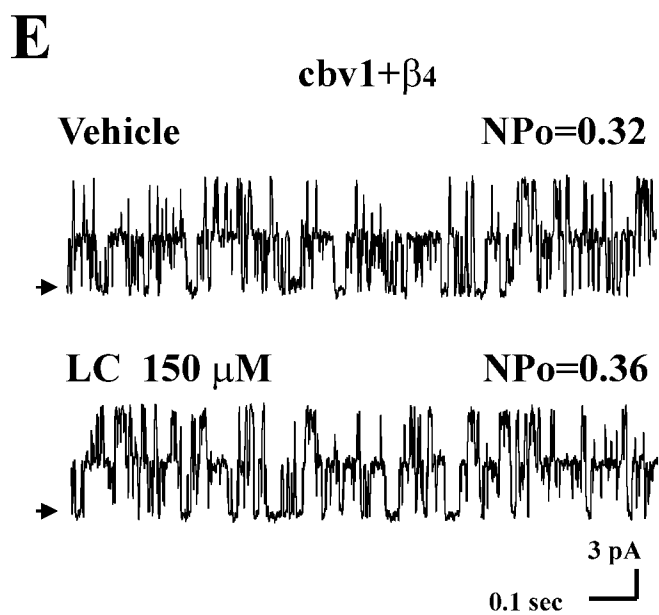
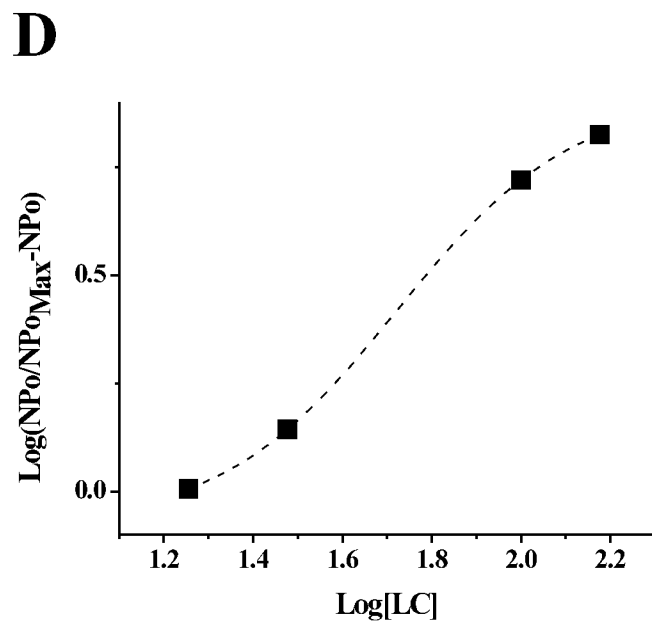
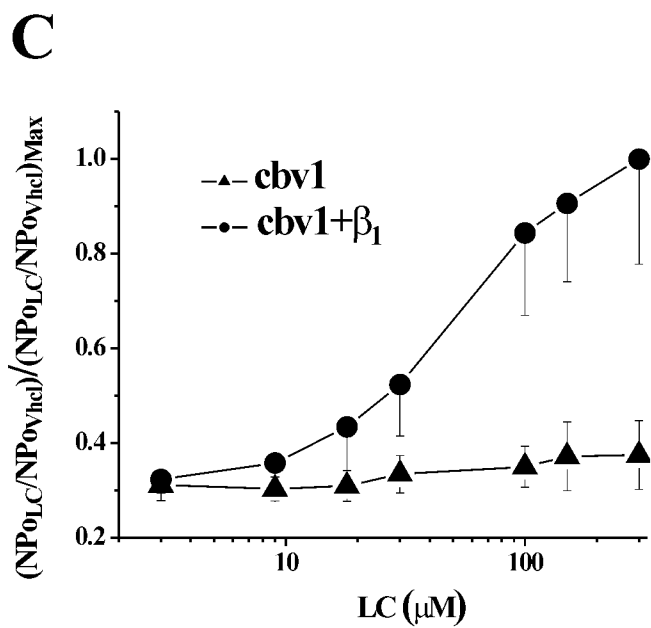
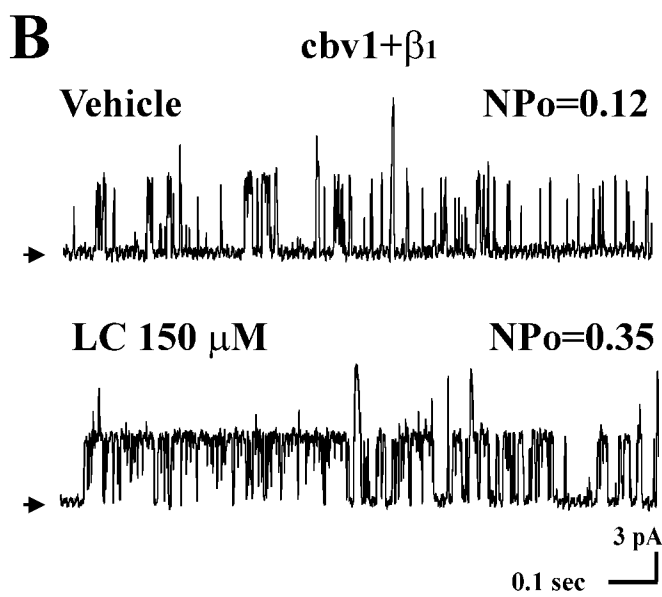
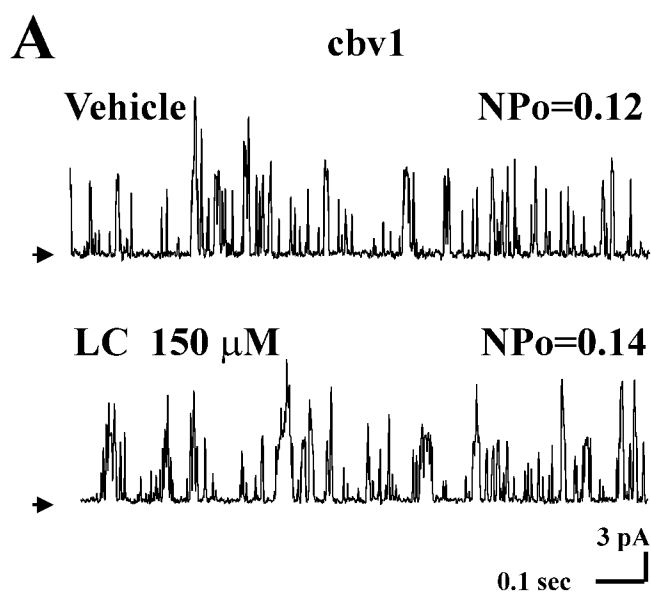
**Table 1. Characteristics of lithocholic acid- vs. vehicle-induced vasodilation.**

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

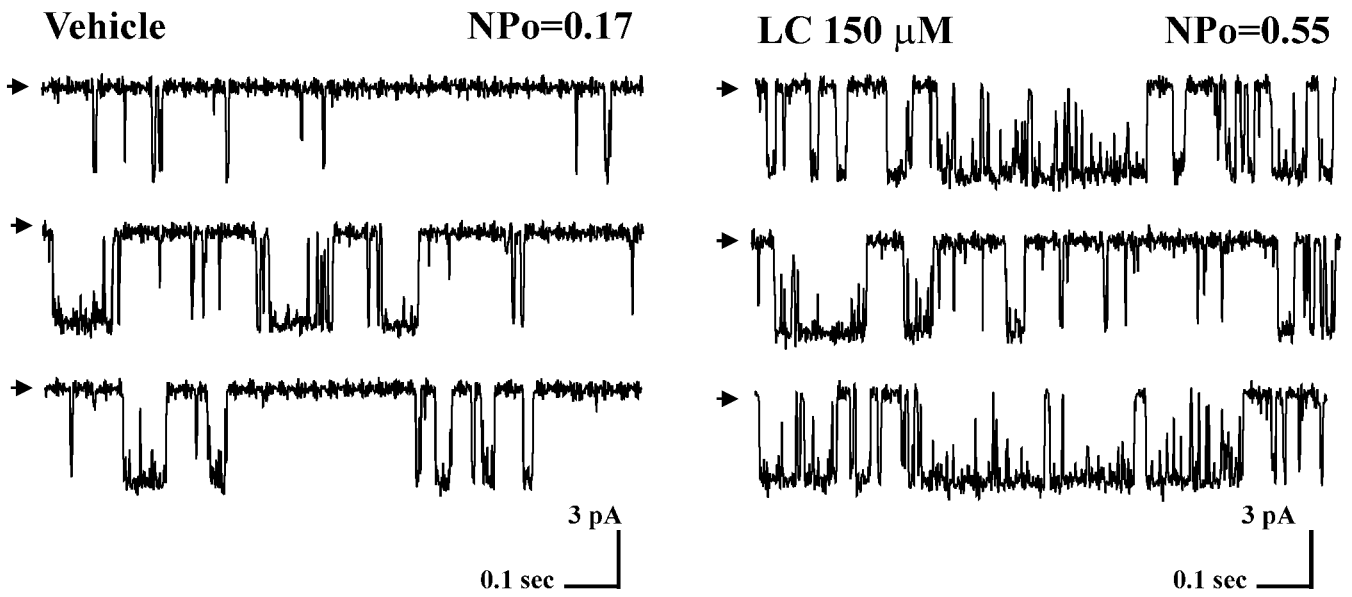
\*\*Significantly different from control, vehicle-containing solution ( $p < 0.01$ ) (paired Student's *t*-test).

**A****B****C****D****Figure 1**

**A****B***Figure 2*

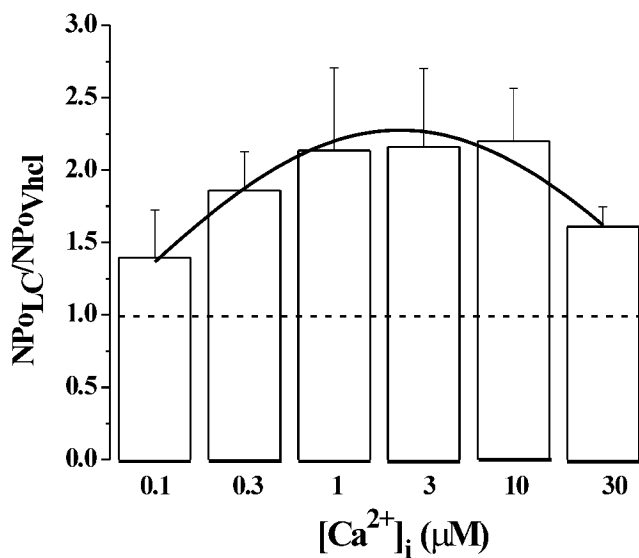
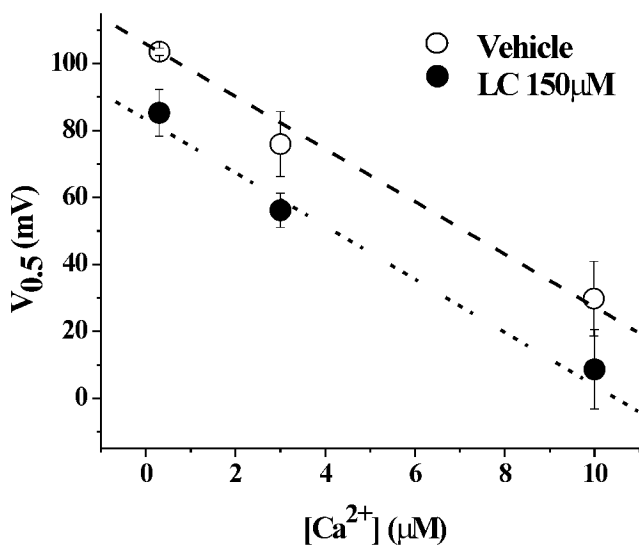


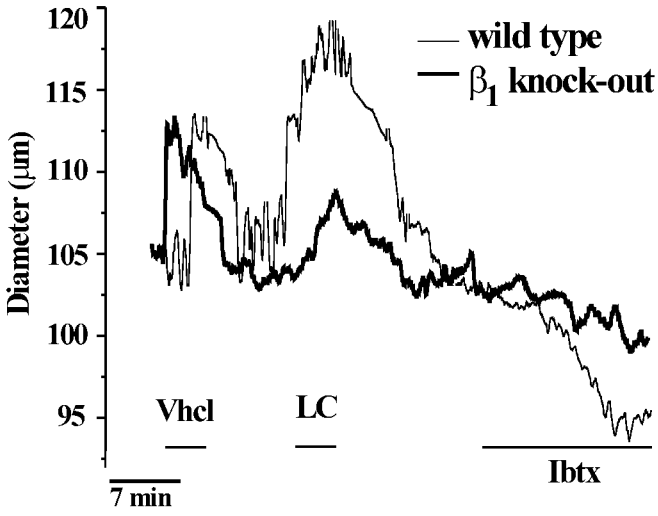
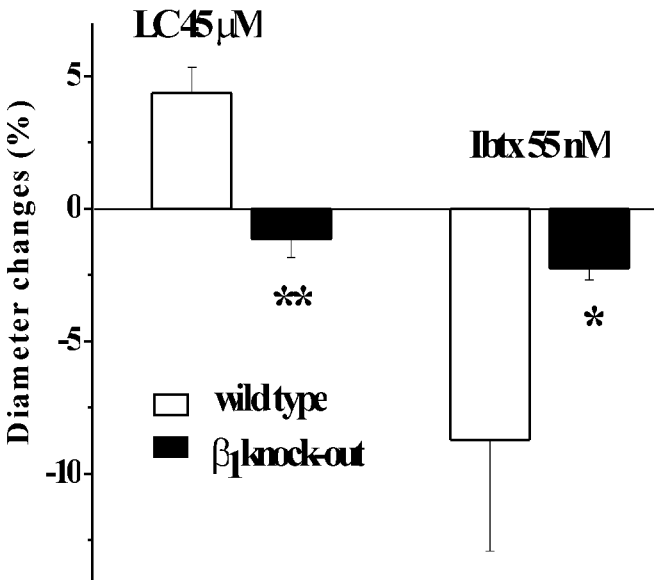
**Figure 3**

**A****B**

	Vehicle (Po=0.17)	LC 150 $\mu$ M (Po=0.55)
$\tau$ open, msec (% of total distribution)	$\tau$ fast 1.69 $\pm$ 0.013 (51%)	$\tau$ fast 2.28 $\pm$ 0.37 (43%)
	$\tau$ slow 14.97 $\pm$ 2.19 (49%)	$\tau$ slow 18.25 $\pm$ 1.89 (57%)
<b>Mean open time</b>	<b>8.14<math>\pm</math>1.14 msec</b>	<b>11.32<math>\pm</math>1.24 msec</b>
$\tau$ close, msec (% of total distribution)	$\tau$ fast 1.66 $\pm$ 0.24 (56%)	$\tau$ fast 1.45 $\pm$ 0.16 (66%)
	$\tau$ slow 74.57 $\pm$ 10.10 (44%)	$\tau$ slow 38.00 $\pm$ 6.18 (34%)
<b>Mean close time</b>	<b>33.74<math>\pm</math>4.57 msec</b>	<b>14.06<math>\pm</math>2.21 msec</b>

*Figure 4*

**A****B***Figure 5*

**A****B***Figure 6*