

**Cerebrovascular dilation via selective targeting of the cholane steroid-recognition site in  
the BK channel  $\beta$ 1 subunit by a novel nonsteroidal agent**

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**Running title:** Pharmacological vasodilation *via* the BK  $\beta$ 1 steroid site

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**Non-standard Abbreviations and Acronyms:** **aCSF**, artificial cerebral spinal fluid; **4-AP**, 4-aminopyridine; **BK**, large-conductance,  $\text{Ca}^{2+}$ -activated  $\text{K}^+$ ; **CRC**, concentration response curve, **DHS-I**, dehydrosoyasaponin-I; **DM**, dissociation medium; **DMSO**, dimethyl sulfoxide; **HENA**, sodium 3-hydroxyolean-12-en-30-oate; **I/O**, inside-out;  **$\text{K}_v$** , voltage-gated  $\text{K}^+$ ; **LCA**, lithocholic acid; **N**, number of functional channels in the membrane patch; **PAD**, pial arteriolar diameter;  **$P_o$** , channel open probability; **PSS**, physiological saline solution; **PT**, pentacyclic triterpenes; **SSS**, sodium saline solution; **TM**, transmembrane

## Abstract

The Ca<sup>2+</sup>/voltage-gated K<sup>+</sup> (BK) channel  $\beta$ 1 subunit is particularly abundant in vascular smooth muscle. By determining their phenotype, BK  $\beta$ 1 allows BK channels to reduce myogenic tone, facilitating vasodilation. The endogenous steroid lithocholic acid (LCA) dilates cerebral arteries *via* BK channel activation, which requires recognition by a BK  $\beta$ 1 site that includes Thr169. Whether exogenous nonsteroidal agents can access this site to selectively activate  $\beta$ 1-containing BK channels and evoke vasodilation remain unknown. We performed chemical structure database similarity search using LCA as template, and a two-step reaction to generate sodium 3-hydroxyolean-12-en-30-oate (HENA). HENA activated BK (cbv1+ $\beta$ 1) channels cloned from rat cerebral artery myocytes with potency (EC<sub>50</sub>=53  $\mu$ M) similar to, and efficacy ( $\times$ 2.5 potentiation) significantly greater than that of LCA. This HENA action was replicated on native channels in rat cerebral artery myocytes. HENA failed to activate channels made of cbv1+ $\beta$ 2,  $\beta$ 3,  $\beta$ 4, or  $\beta$ 1T169A, indicating this drug selectively targets  $\beta$ 1-containing BK channels *via* the BK  $\beta$ 1 steroid-sensing site. HENA (3-45  $\mu$ M) dilated rat and C57BL/6 mouse pressurized cerebral arteries. Consistent with electrophysiological results, this effect was larger than that of LCA. HENA failed to dilate arteries from *KCNMB1* K/O mouse, underscoring BK  $\beta$ 1's role in HENA action. Finally, carotid artery-infusion of HENA (45  $\mu$ M) dilated pial cerebral arterioles *via* selective BK channel-targeting. In conclusion, we have identified for the first time a nonsteroidal agent that selectively activates  $\beta$ 1-containing BK channels by targeting the steroid-sensing site in BK  $\beta$ 1 and renders vasodilation.

## Introduction

A widespread feature in ion channel organization in excitable tissues is the association of the ion channel-forming protein(s) with accessory subunits. These accessory proteins cannot form ion channels themselves but modify the ion current phenotype, including its pharmacology. Moreover, tissue-specific expression of channel subunits allows the resulting hetero-oligomeric channel complex to regulate physiology in a tissue-specific manner (Orio et al., 2002; Yan and Aldrich, 2012). Large conductance, voltage- and  $\text{Ca}^{2+}$ -gated  $\text{K}^+$  (BK) channels result from the association of four identical  $\alpha$  (slo1) subunits, which are ubiquitously distributed (Ghatta et al., 2006; Salkoff et al., 2006). In most tissues, however,  $\alpha$  homotetramers are accompanied by small, 2TM-spanning accessory ( $\beta$ 1-4) subunits that are encoded by four separate genes: *KCNMB1-4* (Orio et al., 2002). Remarkably, *KCNMB1* expression is highly tissue-specific, being highly abundant in the smooth muscle, including arterial myocytes (Brenner et al., 2000a, 2000b; Orio et al., 2002).

BK channels are critical determinants of artery myogenic tone: upon activation, they generate outward currents that counteract depolarization-induced  $\text{Ca}^{2+}$  entry, limiting constriction and favoring artery dilation (Jaggar et al., 1998; Ghatta et al., 2006). BK  $\beta$ 1 subunits increase the apparent  $\text{Ca}^{2+}$ -sensitivity of the arterial smooth muscle BK channel, so that this channel may be activated at voltage and  $\text{Ca}^{2+}$  levels reached in contracting myocytes and thus, exert its negative feed-back on myocyte contraction (Jaggar et al., 1998; Rusch, 2009). The critical role of BK  $\beta$ 1 in controlling myogenic tone and vasomotion has been underscored by several basic research and clinical findings. For example, *KCNMB1* K/O mice are characterized by uncoupling between vasodilating, RyR-generated  $\text{Ca}^{2+}$ -sparks and BK channel-generated Spontaneous Transient Outward Currents (STOCs), which leads to increased myogenic tone, and

systemic hypertension (Plüger et al., 2000). It should be mentioned, however, that *KCNMB1* genetic ablation also results in  $K^+$  retention and hyperaldosteronism, major contributors to the increase in blood pressure found in the *KCNMB1* K/O mouse (Holtzclaw et al., 2011). Also in a mouse model, down-regulation of BK  $\beta$ 1 by NFATc3 activation contributes to systemic hypertension (Nieves-Cintrón et al., 2007). In humans, the gain-of-function Glu65Lys substitution in BK  $\beta$ 1 has been associated with low prevalence of diastolic (Fernández-Fernández et al., 2004; Nielsen et al., 2008) and systolic hypertension (Nielsen et al., 2008).

The central role of BK  $\beta$ 1 in limiting vascular smooth muscle contraction, its scarce expression in tissues other than smooth muscle, and its poor identity with other membrane proteins, raised the expectation that ligands that selectively target this subunit could evoke effective vasodilation *via* smooth muscle BK channel activation while targeting neither BK channels in other tissues nor other receptors.

The quest for newer vasodilators acquires particular relevance when considering the cerebral circulation. Cerebral vasoconstriction underlies numerous pathological conditions, including cerebral vasospasm, ischemia after aneurysmal subarachnoid hemorrhage (Jordan and Nyquist, 2010), the reversible cerebral vasoconstriction syndrome associated with non-aneurysmal subarachnoid hemorrhage, pregnancy and exposure to certain drugs (Sattar et al., 2010), post-traumatic cerebral vasospasm following traumatic brain injury (Shahlaie et al., 2009), and abrupt-onset severe headaches (Ju and Schwedt, 2010). In addition, cerebral vasoconstriction and hypertension may coexist (Sekine et al., 2012). In spite of the high incidence and prevalence of cerebrovascular conditions associated with vasoconstriction, biomedical research has largely failed to provide effective and safe cerebrovascular dilators (Dorsch, 2011; Etminan et al., 2011).

A variety of physiologically-relevant cholane steroids has been reported to activate smooth muscle BK channels (Dopico et al., 2002; Bukiya et al., 2007; 2008a). Moreover, the most effective, lithocholic acid (LCA), evokes a robust cerebral artery *in vitro* dilation *via* activation of  $\beta$ 1 subunit-containing BK channels (Bukiya et al., 2007). Recently, the LCA-recognition site was mapped to the BK  $\beta$ 1 TM2 region. This steroid-recognition site includes Thr169 (Bukiya et al., 2011), a residue that is unique to BK  $\beta$  of type 1 (Brenner et al., 2000a), which likely explains the fact that BK  $\beta$ 2-4 fail to substitute for  $\beta$ 1 in providing LCA-sensitivity to BK channels (Bukiya et al., 2009a). Due to their steroidal nature, LCA and related cholanes exert numerous biological effects outside the vascular bed by interacting with many proteins, including the G-protein-coupled receptor BG37 (Maruyama et al., 2002), vitamin D (Makishima et al., 2002), ryanodine and IP3 receptors (Gerasimenko et al., 2006), cytosolic steroid-binding proteins, membrane transporters, and transcription factors (Modica and Moschetta, 2006). The resulting varied pharmacological profile would likely cause widespread side effects, were LCA and cholanes incorporated into clinical practice as vasodilators.

We hypothesized that LCA can be used as a template for the discovery of *nonsteroidal* selective activators of  $\beta$ 1 subunit-containing BK channels and thus, effective vasodilators. Here, we test and support this hypothesis by combining chemical database similarity search, organic synthesis, computational modeling, point mutagenesis, patch-clamp electrophysiology on recombinant BK channels cloned from rat cerebral artery myocytes and their native counterparts in the myocyte membrane, the *KCNMB1* K/O mouse model, and arterial diameter determinations both in isolated, pressurized arteries and *in vivo*, using a closed cranial window.

## Materials and Methods

**Ethical Aspects of Research.** The care of animals and experimental protocols were reviewed and approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center, which is an Association for Assessment and Accreditation of Laboratory Animal Care-accredited institution.

**Database Search and Computational Studies.** Similarity search based on LCA structure was performed throughout the Hit2Lead.com database using a 70% similarity threshold. This screening rendered methyl 3-hydroxyolean-12-en-30-oate (compound ID#5808244) as a hit lead. Stochastic conformational search with the dielectric constant set to zero was performed to determine the lowest energy conformation of the LCA and hydrolyzed methyl 3-hydroxyolean-12-en-30-oate structures. Stochastic flexible alignment of hydrolyzed methyl 3-hydroxyolean-12-en-30-oate structure with the lowest energy conformation of LCA was performed. Both conformational search and flexible alignment were run using built-in functions in MOE 2006.08 (Chemical Computing Group, Montreal, Canada).

**Chemistry.** 3'-hydroxy-olean-12-en-29-oic acid methyl ester (compound **1**) was hydrolyzed to obtain 3'-hydroxy-olean-12-en-29-oic acid (compound **2**) (**Supplemental Figure 1**). Purified acid (**2**) was further converted to the corresponding sodium salt (compound **3**) to improve solubility for probing of biological activities.

*Experimental description:* 3'-hydroxy-olean-12-en-29-oic acid methyl ester (compound **1**) was purchased from -Hit2Lead.com and was used for hydrolysis without further purification. Organic solvents used for reaction as well as purification were purchased from Sigma-Aldrich

(St. Louis, MO) and used without further purification. Characterization of the 3'-hydroxy-olean-12-en-29-oic acid (**2**) has been conducted using proton ( $^1\text{H}$ ) and ( $^{13}\text{C}$ ) carbon NMR spectra (Varian 500-MHz spectrometer; Varian, Inc., Palo Alto, CA) along with mass spectral data (Bruker-HP Esquire-LC spectrometer, Bruker, Billerica, MA). Yields refer to purified products.

*Preparation of 3'-hydroxy-olean-12-en-29-oic acid ( compound 2;*

*2S,4aS,6aS,6bR,8aR,10S,12aR,12bR,14bR)-10-hydroxy-2,4a,6a,6b,9,9,12a-heptamethyl-1,2,3,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,12b,13,14b-icosahydricene-2-carboxylic acid* ): 3'-hydroxy-olean-12-en-29-oic acid methyl ester (0.085 mM, 40 mg) was dissolved in 10 mL of ethanol. After adding 300 mg KOH dissolved in 2 mL of water the mix was refluxed for nearly 8 hours. The reaction mixture was then cooled and carefully acidified with diluted HCl. The mixture was extracted with chloroform. Chloroform layer was dried over anhydrous  $\text{Na}_2\text{SO}_4$ . Chloroform was removed under reduced pressure. The crude residue was purified by flash chromatography (EtOAc:hexanes; 1:9).

*Yield 34%;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  5.32 (t,  $J = 10.0$  Hz, 1H), 3.25 (m, 1H,  $3\beta\text{-OCH}$ ), 1.78-2.05 (m, 6H), 1.72-1.48 (m, 7H), 1.46-1.13 (m, 14H, 2 $\text{CH}_3$  groups were merged in the multiplet), 1.08-0.93 (m, 10H, 3 $\text{CH}_3$  groups were merged in the multiplet), 0.90-0.72 (m, 8H, 2 $\text{CH}_3$  groups were merged in the multiplet);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  181.20, 143.68, 122.24, 78.10, 54.72, 47.54, 47.15, 43.46, 42.16, 41.03, 39.30, 38.24, 38.10, 37.78, 36.45, 32.17, 31.43, 30.62, 28.08, 27.56, 26.71, 26.46, 25.64, 25.43, 22.95, 17.84, 16.26, 15.01, 14.93; MS (ESI):  $m/z$  455.1 [ $\text{M} - \text{H}$ ]; Anal. Calcd. ( $\text{C}_{30}\text{H}_{48}\text{O}_3$ ).*

*Preparation of sodium salt of 3'-hydroxy-olean-12-en-29-oic acid ( compound 3):* NaOH (1.93 mg) was moistened with a drop of water in a small vial. To this vial, 0.5 mL methanol and 11

mg of pure acid (**2**) were added and stirred for 5 minutes. Methanol was evaporated under reduced pressure, and then residue was finally dried over high vacuum. This residue was referred to as “HENA” and used to test biological activities.

**cDNA Cloning and Transcription.** cDNA cloning and functional characterization of rat cerebral artery myocyte BK channel-forming (cbv1) subunits (AY330293) are described elsewhere (Jaggar et al., 2005). Cbv1 cDNA inserted into pBScMXT vector was linearized with Sall and transcribed using T3 (mMessage-mMachine; Ambion, Austin, TX).

cDNA of rat cerebral artery myocyte BK  $\beta$ 1 subunit (FJ154955) was cloned as described in our previous work (Bukiya et al., 2009a). BK  $\beta$ 2-4, and  $\beta$ 4/ $\beta$ 1 ( $\beta$ 4TM2<sub>1</sub>) chimeric cDNAs were generous gifts from Dr. Ligia Toro (Univ. of California at Los Angeles, Los Angeles, CA). Upon arrival, they were re-cloned into pOx vector. Targeted T169A mutation was introduced into *wt* BK  $\beta$ 1 cDNA inserted in pOX using overlap-extension PCR and the Quickchange kit with pfu polymerase (Agilent Technologies, Santa Clara, CA) following manufacturer’s instructions. Sequence of chimeric cDNA constructs, presence of targeted mutation, and absence of unintended mutations in the PCR-amplified regions of the  $\beta$ 1-4 subunit constructs was verified by automated sequencing (Molecular Resource Center, UTHSC).

Beta1-4 cDNAs inserted into pOX vector were linearized and then transcribed *in vitro* using either T3 or T7 polymerases (mMessage-mMachine; Ambion, Austin, TX).cRNA was dissolved in diethyl polycarbonate-treated water at 10 (cbv1) and 30 ( $\beta$ 1,  $\beta$ 2,  $\beta$ 3,  $\beta$ 4,  $\beta$ 4TM2<sub>1</sub> or  $\beta$ 1T169A) ng/ $\mu$ L; 1  $\mu$ L aliquots were stored at -70°C.

**Oocyte Extraction and cRNA Injection.** *Xenopus laevis* females were purchased from Xenopus Express (Brooksville, FL) and were maintained in artificial pond water on a 12-hour light/dark cycle. In this habitat, they do not show seasonal breeding behavior, so oocytes are available throughout the year. Stages V and VI oocytes were predominantly used, because they transcribe mRNA into channels efficiently. Five frogs were used as oocyte donors. Prior to surgery, frogs were anesthetized by placement on ice following exposure to ethyl 3-aminobenzoate methanesulfonate salt (250 mg/L, pH=7.4). Oocytes were removed and kept in a  $\text{Ca}^{2+}$ -free ND-96 solution (mM): 82.5 NaCl, 2 KCl, 1  $\text{MgCl}_2$ , 5 HEPES, pH 7.5, containing 2 mg/mL collagenase (type IV, Sigma, St. Louis, MO), at room temperature on shaker (60 oscillations per minute) for 15 min to remove the follicular layer. After defolliculation, oocytes were transferred to a  $\text{Ca}^{2+}$ -containing ND-96 saline (mM): 82.5 NaCl, 2 KCl, 1.8  $\text{CaCl}_2$ , 5 HEPES, pH 7.4, supplemented with 2.5 mM sodium pyruvate and 2 mg/mL gentamicin. Injection of mRNA into oocyte cytoplasm was conducted using a Drummond micropipette modified for microinjection (Drummond Scientific Co., Broomall, PA). The interval between cRNA injection and patch-clamping was 36-48 hours. During this time, injected oocytes were kept at 15°C.

**Rat Cerebral Artery Myocyte Isolation.** Middle and basilar cerebral arteries were isolated from adult male Sprague-Dawley rats ( $\approx 250$  g). Ten rats were used as myocyte donors. One animal per day was decapitated using a guillotine. 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) with the following composition (mM): 0.16  $\text{CaCl}_2$ , 0.49 EDTA, 10 HEPES, 5 KCl, 0.5  $\text{KH}_2\text{PO}_4$ , 2  $\text{MgCl}_2$ , 110 NaCl, 0.5  $\text{NaH}_2\text{PO}_4$ , 10

NaHCO<sub>3</sub>, 0.02 phenol red, 10 taurine, 10 glucose. Each artery was cut into 1- to 2-mm long rings (up to 30 rings/experiment). Individual myocytes were enzymatically isolated. For this purpose, rat arterial rings were put in 3 ml DM containing 0.03% papain, 0.05% bovine serum albumin (BSA), and 0.004% dithiothreitol at 37°C for 15 min in a polypropylene tube and incubated in a shaking water bath at 37 °C and 60 oscillations/min for 15 min. Then, the supernatant was discarded and the tissue transferred to a polypropylene tube with 3 mL DM containing 0.06% soybean trypsin inhibitor (STI), 0.05% BSA, and 2% collagenase (26.6 units/mL). The tube was incubated again in a shaking water bath at 37°C and 60 oscillations/min for 15 minutes. Finally, artery tissue pellet was transferred into a tube with 3 ml of DM containing 0.06% soybean trypsin inhibitor. Tissue-containing DM was pipetted using a series of borosilicate Pasteur pipettes having fire-polished, diminishing internal diameter tips. The procedure rendered a cell suspension containing relaxed, individual myocytes ( $\geq 5$  myocytes/field using a 20X objective) that could be identified under an Olympus IX-70 microscope (Olympus American Inc., Woodbury, NY). The cell suspension was stored in ice-cold DM containing 0.06% STI and 0.06% BSA. Myocytes were used for electrophysiology up to 4 hours after isolation.

**Electrophysiology Data Acquisition and Analysis.** Before recordings, oocytes were placed into a dish containing a hypertonic solution (mM): 200 K<sup>+</sup> aspartate, 20 KCl, 1 MgCl<sub>2</sub>, 10 ethylene glycol-bis( $\beta$ -aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 10 HEPES, pH 7.4, for 10 minutes. With this treatment the oocytes shrink, allowing the removal of the vitelline layer with forceps and exposing the oocyte membrane for subsequent patch recording. Then the oocytes were placed back into ND-96 saline (in this case without gentamicin; for composition,

see above) for 10 to 15 minutes before ion current recording. Currents were recorded from excised, inside-out (I/O) patches. For experiments with oocytes, both bath and electrode solutions contained (mM): 130 K<sup>+</sup> gluconate, 5 EGTA, 2.28 MgCl<sub>2</sub>, 15 HEPES, 1.6 HEDTA, 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>. When desired free Ca<sup>2+</sup> did not exceed 1 μM, HEDTA was omitted from the solution, and final MgCl<sub>2</sub> was set to 1 mM. In all cases, 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) (Dopico, 2003).

Patch-clamp electrodes were pulled from glass capillaries (Drummond Scientific Co., Broomall, PA). Immediately before recording, the tip of the electrode was fire-polished on a microforge (World Precision Instr., Sarasota, FL) to give resistances of 8 to 10 MΩ when filled with extracellular solution (for composition, see above). An agar bridge with gluconate or chloride as the main anion was used as ground electrode for oocyte and myocyte recordings, accordingly. 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 patch cytosolic side using a pressurized, automated DAD12 system (ALA Scientific Instruments, Farmingdale, NY) via a micropipette tip with an internal diameter of 100 μm. Experiments were carried out at room temperature (20-22°C).

Ionic current was recorded using an EPC8 amplifier (HEKA; Lambrecht, Germany) at 1 kHz. Data were digitized at 5 kHz using a Digidata 1320A A/D converter and pCLAMP 8.0 (Molecular Devices, Sunnyvale, CA). The product of number of channels in the patch (N) and channel open probability (Po) was used as an index of channel steady-state activity. NPo was

obtained using a built-in option in Clampfit 9.2 (Molecular Devices, Sunnyvale, CA) from  $\geq 30$  sec of gap-free recording under each condition.

Macroscopic currents were evoked from a holding potential of 0 mV by 200 ms-long, 10 mV steps ranging from -150 to +150 mV. Current amplitude was averaged within 100-150 ms after the start of the depolarizing step. Macroscopic conductance  $G/G_{\max}$ -V plots were fitted to a Boltzmann function of the type  $G(V) = G_{\max} / (1 + \exp[-(V - V_{1/2})/k])$ . Boltzmann fitting routines were run using the Levenberg-Marquardt algorithm to perform nonlinear least squares fits.

**Cerebral Artery Diameter Measurement.** Adult male Sprague-Dawley rats ( $\approx 250$  g; 10 animals) and 8 to 12 week-old male *KCNMB1* K/O (5 animals) and C57BL/6 control (6 animals) mice were decapitated using a guillotine and sharp scissors, respectively. Middle cerebral arteries were isolated on ice under microscope (Nikon SMZ645; Nikon, Tokyo, Japan) from rat or mouse brain and cut into 1 to 2 mm-long segments. Endothelium was removed by passing an air bubble into the vessel lumen for 90 seconds prior to vessel cannulation. A segment was cannulated at each end in a temperature-controlled, custom-made perfusion chamber. Using a Dynamax RP-1 peristaltic pump (Rainin Instruments, Inc., Oakland, CA), the chamber was continuously perfused at a rate of 3.75 ml/minute with PSS (mM): 119 NaCl, 4.7 KCl, 1.2  $\text{KH}_2\text{PO}_4$ , 1.6  $\text{CaCl}_2$ , 1.2  $\text{MgSO}_4$ , 0.023 EDTA, 11 glucose, 24  $\text{NaHCO}_3$ . PSS was equilibrated at pH 7.4 with a 21/5/74% mix of  $\text{O}_2/\text{CO}_2/\text{N}_2$  and maintained at 35-37°C. Arteries were observed with a CCD camera (Sanyo VCB-3512T; Sanyo Electric Co., Moriguchi, Japan) attached to an inverted microscope (Nikon Eclipse TS100; Nikon, Tokyo, Japan). The artery external wall diameter was measured using the automatic edge-detection function of IonWizard software (IonOptics, Milton, MA) and digitized at 1 Hz using a personal computer. Steady-state changes

in intravascular pressure were achieved by elevating an attached reservoir filled with PSS and were monitored using a pressure transducer (Living Systems Instr.; Burlington, VT). Arteries were first incubated at an intravascular pressure of 10 mm Hg for 10 minutes. Then, intravascular pressure was increased to 60 mmHg and held steady throughout the experiment to develop and maintain arterial myogenic tone. Drugs were dissolved to make stock solutions, diluted in PSS to final concentration, and applied to the artery via chamber perfusion. The effect of drug applications was evaluated at the time it reached a maximal, steady level. Absence of endothelium was confirmed by the absence of vessel's response to endothelium-dependent (10  $\mu$ M acetylcholine) vasodilator after artery pressurization (Liu et al., 2004; Bukiya et al., 2007).

**Pial Arteriolar Diameter Measurements.** Adult male Sprague-Dawley rats ( $\approx$ 250-300 g; 20 animals) were anesthetized with ketamine hydrochloride (33 mg/kg i.m.) and acepromazine (3.3 mg/kg i.m.). Catheters inserted into the carotid artery were used to infuse drugs of interest; drugs were dissolved to final concentration in sodium saline solution (0.9% NaCl) (SSS). Body temperature was maintained at 37–38°C with a heating pad. The animals were equipped with a custom-built stainless steel cranial window that allowed: 1) measurement of pial arteriolar diameter (PAD) with a videomicrometer coupled to a television camera; 2) delivery of drugs directly to the brain surface through the ports of the window. The space under the window was filled with artificial cerebrospinal fluid (aCSF) that consisted of (mM): 3.0 KCl, 1.5 MgCl<sub>2</sub>, 1.5 CaCl<sub>2</sub>, 132 NaCl, 6.6 urea, 3.7 dextrose, and 24.6 NaHCO<sub>3</sub>. This solution was equilibrated with 6% CO<sub>2</sub>/6% O<sub>2</sub>/88% N<sub>2</sub> to pH 7.3–7.35 at 37°C. Pial arterioles (50–100  $\mu$ m in external diameter) were used to test vascular reactivity. Control PAD values were measured over a 10-min period under basal conditions. LCA and HENA stock solutions were diluted in SSS to a

final concentration of 45  $\mu$ M and 1 mL of this solution was infused into the cerebral circulation via the carotid artery. Stock solution of paxilline in DMSO was diluted in aCSF to a final concentration of 1  $\mu$ M. Paxilline and 4-AP (0.8 mM in aCSF) were applied topically on the surface of the brain.

**Chemicals.** 5 $\beta$ -cholanic acid-3 $\alpha$ -ol (lithocholic acid) was purchased from Steraloids (Newport, RI) and methyl 3-hydroxyolean-12-en-30-oate was purchased from Hit2Lead.com (ChemBridge Corp., San Diego, CA). All other chemicals were purchased from Sigma (St. Louis, MO). On the day of the experiment, LCA or HENA were initially dissolved in DMSO to render stock solutions of 333mM. Stocks were sonicated for 30 minutes and then further diluted with either electrophysiology bath recording, PSS or SSS solution to final concentration. In all experiments DMSO-containing solution was used as control perfusion. Concentration of DMSO in “control” matched the corresponding amount of DMSO in LCA- or HENA-containing solution.

**Data Analysis.** Final plotting, fitting and statistical analysis of the data were conducted using Origin 8.5.1 (OriginLab, Northampton, MA) and InStat 3.0 (GraphPad, La Jolla, CA). Statistical analysis was conducted using either one-way ANOVA and Bonferroni’s multiple comparison test or paired Student’s t-test, according to experimental design. Significance was set at  $P < 0.05$ . Data are expressed as mean $\pm$ SEM; n=number of patches/arteries/pial arterioles. Each patch was obtained from a different oocyte/myocyte, and each pressurized artery/PAD measurement was obtained from a separate animal.

## Results

**Sodium 3-hydroxyolean-12-en-30-oate (HENA) is a novel, effective non-steroid activator of  $\beta$ 1 subunit-containing recombinant BK channels.** Among 18 cholane steroids tested, LCA was the most effective activator of  $\beta$ 1-containing BK channels (Dopico et al., 2002; Bukiya et al., 2008a). Thus, in search for novel selective activators of these channels we took the LCA molecule (**Fig. 1A, top structure**) as the template for a similarity search, and screened the Hit2Lead.com database of chemical compounds. The screening yielded methyl 3-hydroxyolean-12-en-30-oate (ID #5808244; **Fig. 1A, bottom structure**) as the lead compound. This compound is not a steroid, yet contains all structural features previously identified as necessary for cholane steroid activation of BK channels: C3-hydroxyl, hydrophobic nucleus and polar lateral chain (Dopico et al., 2002; Bukiya et al., 2008a; 2012). The highly hydrophobic methyl 3-hydroxyolean-12-en-30-oate required dissolution in pure DMSO before further dissolution in saline solutions for biological evaluation. The resulting DMSO concentration in saline solution exceeded 0.5%, which is known to lead to apoptosis (Qi et al., 2008). Thus, to improve active compound solubility and bolster structural similarity between methyl 3-hydroxyolean-12-en-30-oate and LCA, we performed a 2-step organic chemical reaction to render sodium 3-hydroxyolean-12-en-30-oate (HENA) (**Fig. 1A, bottom structure; Supplemental Figure 1**). With a carboxylate similar to that of LCA ( $pK_a \sim 5.5$ ), the HENA molecule is expected to remain significantly ionized at physiological  $pH \approx 7.3-7.4$ . Thus, flexible alignment of ionized LCA and HENA molecules showed high degree of overlap at both their C3-hydroxyl and carboxyl ends (**Fig. 1B**). Most important, HENA was able to adopt a bean-like shape, which is critical for BK channel activation by LCA and related cholane steroids (Dopico et al., 2002; Bukiya et al.,

2008a). Therefore, HENA exhibits all main structural features considered necessary for BK channel activation *via* the BK  $\beta 1$  subunit cholane steroid site (Bukiya et al., 2008a; 2011).

To determine HENA's ability to activate BK channels, we first studied ligand action on recombinant cbv1+ $\beta 1$  channels heterologously expressed in *Xenopus* oocytes. We cloned both BK channel-forming cbv1 (AY330293) and accessory  $\beta 1$  (FJ154955) subunits from freshly isolated rat cerebral artery myocytes, the resulting recombinant BK complex representing an ideal model of the native BK channel (Bukiya et al., 2009a). In our experiments, we used inside-out (I/O) patches with the membrane potential and free  $Ca^{2+}_i$  set at values similar to those reported in the rat cerebrovascular myocyte during contraction (-40 to -30 mV and 10  $\mu M$ ), that is, when BK channel activity plays a critical role in limiting depolarization-induced  $Ca^{2+}$ -entry and thus, further contraction (Jaggar et al., 1998; Knot and Nelson, 1998; Pérez et al., 2001). After excision, each patch was exposed to control (solvent-containing) solution and BK activity was continuously recorded for no less than 30 seconds. Application of LCA-containing (1-1,000  $\mu M$ ) solution reversibly increased NPo in a concentration-dependent fashion:  $EC_{50}=46.9\pm 4.1$   $\mu M$ ,  $E_{max}\sim 300$   $\mu M$  (**Figs. 2A, D, E**). These values are similar to those reported with LCA on this system and attributed to a direct interaction between LC monomers and the BK channel proteins (Bukiya et al., 2007). Application of HENA-containing (1-1,000  $\mu M$ ) solution also reversibly increased NPo in a concentration-dependent manner, with potency similar to that of LCA:  $EC_{50}=53.4\pm 3.9$   $\mu M$  and  $E_{max}\sim 300$   $\mu M$  (**Figs. 2B, D, E**). For both LCA and HENA, drug potentiation of BK channel activity did not depend on basal, pre-drug channel activity (**Fig. 2C**). At every concentration tested, however, HENA was more effective than LCA in increasing BK NPo (**Fig. 2D**), with NPo in presence of HENA  $E_{max}$  reaching x2.5 times that of control.

It is noteworthy that HENA-induced BK channel activation remained fairly constant within a wide voltage range (-150 to +150 mV), as documented by a parallel leftward shift in G/Gmax plots from macroscopic  $cbv1+\beta1$  currents in presence vs. absence of HENA (**Figs. 3A, B**). On the other hand, HENA-induced BK channel activation was identical at submicromolar (0.3  $\mu\text{M}$ ) and micromolar (10  $\mu\text{M}$ ) levels of free  $\text{Ca}^{2+}_i$  (**Figs. 4A, B**). Collectively, our results demonstrate that HENA-induced BK channel activation is voltage-independent and observed at levels of  $\text{Ca}^{2+}_i$  found in the resting and contracting cerebral artery myocyte (Pérez et al., 2001).

Considering that: 1) HENA presents a high structural similarity with LCA (**Fig. 1**), and 2) LCA selectively activates  $\beta1$  subunit-containing BK channels (Bukiya et al., 2009b), we decided to determine whether HENA-induced BK channel activation required the presence of BK  $\beta1$  subunits. We performed electrophysiological recordings in the I/O membrane patches from *Xenopus* oocytes expressing heteromeric  $cbv1+$  either  $\beta2$ ,  $\beta3$ , or  $\beta4$  subunits, or homomeric  $cbv1$  channels under identical conditions of transmembrane voltage ( $V_m=-40\text{mV}$ ) and  $\text{Ca}^{2+}_i$  (10  $\mu\text{M}$ ). In all cases, the resulting ion current phenotype was confirmed by characteristic BK current kinetics (**Supplemental Figure 2**) or distinctive pharmacological features, as we previously described in detail (Bukiya et al., 2009a). In contrast to their  $cbv1+\beta1$  counterparts, homomeric  $cbv1$  channels were consistently resistant to HENA, even when this compound was tested at 150  $\mu\text{M}$  (**Figs. 5A, bottom right panel, B**), which corresponds to  $\text{EC}_{90}$  for HENA activation of  $\beta1$ -containing BK complexes. Thus, as previously shown for the steroid LCA (Bukiya et al., 2009a), sensitivity of BK channels to the nonsteroid HENA requires the presence of functional BK  $\beta1$  subunits.

Moreover, heteromers made of  $cbv1 +$  either  $\beta2$ ,  $\beta3$  or  $\beta4$  subunits were all HENA-resistant (**Figs. 5A, bottom left and top panels, B**). Thus, as previously found for LCA (Bukiya et al.,

2009b), BK  $\beta$ 2,  $\beta$ 3 and  $\beta$ 4 fail to substitute for  $\beta$ 1 in conferring HENA sensitivity to BK channels. Collectively, the qualitative and quantitative similarities between HENA and LCA action on recombinant cerebrovascular BK channels seem to indicate that BK  $\beta$ 1 behaves as a specific sensor of HENA, likely involving the cholane steroid-recognizing site recently identified in this protein (Bukiya et al., 2011).

**Sodium 3-hydroxyolean-12-en-30-oate activates BK channels *via* cholane steroid-sensing site on BK  $\beta$ 1 subunit TM2 domain.** Based on the fact that the  $\beta$ 1, but not  $\beta$ 4, subunit confers HENA sensitivity to the BK channel, and that LCA exerts its effect *via* transmembrane domain 2 (TM2) of  $\beta$ 1, we used chimeric  $\beta$ 1/ $\beta$ 4 subunits to identify the BK  $\beta$ 1 protein region targeted by HENA. In the chimeric construct termed  $\beta$ 4TM2<sub>1</sub>, the BK  $\beta$ 4 TM2 domain was substituted by the TM2 region from BK  $\beta$ 1. The presence of functional  $\beta$ 4TM2<sub>1</sub> co-expressed with cbv1 was confirmed by the resistance of the current to iberiotoxin (100 nM) block, which is due to the presence of the BK  $\beta$ 4 extracellular loop region (Meera et al., 2000; Bukiya et al., 2008b).

Under experimental conditions similar to those used to probe HENA on *wt*  $\beta$ 1+cbv1, 45  $\mu$ M HENA applied to the cytosolic side of I/O patches evoked a robust and reversible increase in BK NPo in all cases (**Fig. 6A, C**). Remarkably, this effect was identical to that obtained with HENA and cbv1+*wt*  $\beta$ 1 channels (**Figs. 2 B, D, E**), indicating that  $\beta$ 1 TM2 is the channel region that senses HENA presence rendering activation of BK channels.

The cholane-sensing site in the BK  $\beta$ 1 TM2 domain includes Thr169, a residue that is distinct to BK  $\beta$  of type 1 (Brenner et al., 2000a). Computational modeling shows that this residue forms a hydrogen bond with the LCA C3-hydroxyl and, thus, the T169A substitution fully abolishes LCA sensitivity of BK channels (Bukiya et al., 2008a; 2011). Next, we tested

whether HENA recognition by BK  $\beta$ 1 also required Thr169. Thus, we co-expressed cbv1 with BK  $\beta$ 1T169A and evaluated HENA action under experimental conditions identical to those used to evaluate the nonsteroidal agent on cbv1+ $\beta$ 4TM2<sub>1</sub> and cbv1+ *wt*  $\beta$ 1 channels (see above). In all cases, application of HENA, whether at 45  $\mu$ M (**Fig. 6B, C**) or 150  $\mu$ M, onto I/O membrane patches expressing cbv1+ $\beta$ 1T169A failed to modify BK NPo (n=4 for each HENA concentration; data not shown). These negative data indicate that Thr169, a residue unique to BK  $\beta$  subunits of type 1 and critical for providing cholane steroid sensitivity to  $\beta$ 1-containing BK channels, is also necessary for HENA activation of these channels. This fact strongly suggests that HENA activates  $\beta$ 1-containing BK channels *via* specific interaction with the BK cholane steroid site (see Discussion).

### **Sodium 3-hydroxyolean-12-en-30-oate activates native cerebral artery myocyte BK**

**channels.** To determine whether HENA was able to target  $\beta$ 1-containing BK channels when expressed in their natural membrane, we studied drug action on native BK channels in I/O patches excised from freshly isolated rat cerebral artery myocytes while comparing HENA action to that of LCA (**Fig. 7**). Membrane potential and  $Ca^{2+}_i$  were set at values similar to those obtained in cerebrovascular myocytes during contraction: -40 to -30 mV and 3  $\mu$ M free  $Ca^{2+}_i$  (Knot and Nelson, 1998; Pérez et al., 2001). After excision, the patch was exposed to control (vehicle-containing) solution, and BK NPo was recorded for no less than 30 seconds. Application of HENA-containing (1-300  $\mu$ M) solution reversibly increased NPo in a concentration-dependent fashion, with  $EC_{50}$ =46 $\pm$ 6  $\mu$ M and  $E_{max}$ ~300  $\mu$ M (**Fig. 7B, C**). These values are similar to those reported for HENA activation of recombinant cbv1+ $\beta$ 1 channels expressed in amphibian membranes (see above) and LCA activation of rat cerebrovascular

smooth muscle BK channels (**Fig. 7** and also Bukiya et al., 2007). At HENA  $E_{\max}$ , however, NPo reached 220% of control, which is significantly higher than that reached at LCA  $E_{\max}$  (**Figs. 7B, C**). Thus, as found for recombinant channels, HENA is more efficacious than LCA in activating native cerebrovascular myocyte BK channels.

It is noteworthy that HENA-induced increase in NPo was observed in membrane patches that were excised from the myocyte >5 minutes before applying drug under continuous bath perfusion in the absence of nucleotides. Therefore, HENA action does not require cell integrity or the continuous presence of cytosolic messengers to activate native BK channels from freshly isolated rat cerebral artery myocytes. Overall, qualitative and quantitative similarities in HENA action on recombinant vs. native BK channels suggest that the likely different proteolipid environments in amphibians vs. mammalian cell membranes do not affect HENA activation of  $\beta 1$ -containing BK channels, underscoring that potentiation of BK channel activity is due to a direct interaction between the HENA molecule and the BK  $\beta 1$  protein itself.

**Sodium 3-hydroxyolean-12-en-30-oate evokes dilation of smooth muscle BK  $\beta 1$  subunit-containing cerebral arteries.** To determine the impact of smooth muscle BK channel targeting by HENA on organ function, we evaluated drug action on diameter of pressurized middle cerebral arteries from rats. Vessel dissection, de-endothelization and pressurization are described in Material and Methods. After myogenic tone development at 60 mmHg, arteries were sequentially probed with control (DMSO-containing) and HENA- or LCA-containing solutions. At the end of each experiment, the artery was perfused with  $Ca^{2+}$ -free Physiological Saline Solution (PSS) to assess passive arterial diameter.

As previously reported (Bukiya et al., 2007), the presence of vehicle in the chamber caused a mild and transient dilation of the arteries. This effect disappeared upon washout with solvent-free PSS (**Fig. 8A**). After artery perfusion with PSS, brief ( $\leq 10$  min) application of HENA (3-45  $\mu\text{M}$ ) produced an immediate increase in arterial diameter, which was sustained as far as HENA was present in the perfusing solution, and fully recovered to pre-HENA values following washout of HENA with compound-free PSS (**Fig. 8A**). This HENA action was concentration-dependent, with maximal artery dilation being observed in presence of 45  $\mu\text{M}$  HENA: 95% of passive arterial diameter and an averaged 15% increase in artery diameter from pre-HENA diameter values (**Fig. 8A, B**). This HENA action is expected to evoke a marked increase in cerebral blood flow ( $\sim 45\%$ ) because changes in artery diameter are related to changes in cerebral blood flow by a factor of  $\sim 3$  (Gourley and Heistad, 1984).

Finally, HENA-induced *in vitro* dilation of cerebral arteries was prevented by selective block of BK channels with 1  $\mu\text{M}$  paxilline (**Fig. 8C, D**) (Strøbaek et al., 1996). Collectively, 1) this outcome, 2) the fact that arteries were de-endothelized prior to pressurization, and 3) smooth muscle cells are estimated to account for up to 70% of de-endothelized arterial tissue (Lee, 1995), led us to conclude that HENA-induced cerebral artery dilation is primarily due to activation of BK channels present in the vascular smooth muscle. Consistent with this interpretation, HENA-induced increase in arterial diameter was significantly higher than that evoked by LCA (**Fig. 8A, B**) paralleling HENA vs. LCA differential efficacies of smooth muscle BK channel activation (**Fig. 7**). In spite of their marked structural similarities (**Fig. 1**), HENA and LCA differed in their ability to increase overall  $\text{Ca}^{2+}$  in cerebral artery smooth muscle, with 45  $\mu\text{M}$  LCA being effective and HENA not (**Supplemental Figure 3, Supplemental Methods**) (see Discussion).

To determine whether HENA-induced artery dilation resulted from arterial smooth muscle BK  $\beta$ 1 subunit-targeting by this ligand, we evaluated HENA action on the arterial diameter of de-endothelized, pressurized cerebral arteries from  $\beta$ 1 subunit-lacking (*KCNMB1* K/O) vs.  $\beta$ 1 subunit-containing (*wt* C57BL/6) mice. As found with rat cerebral arteries, 45  $\mu$ M HENA caused a sustained, yet fully reversible, increase in diameter of *wt* mouse cerebral arteries ( $\approx$ 10% from diameter before HENA application) (**Fig. 9A, C**). In sharp contrast, HENA consistently failed to dilate arteries from *KCNMB1* K/O mice (n=5) (**Fig. 9B, C**), indicating that the presence of smooth muscle BK  $\beta$ 1 subunits in cerebral arteries is essential for HENA-induced vasodilation.

**Sodium 3-hydroxyolean-12-en-30-oate causes pial arteriolar dilation *in vivo* via selective activation of BK channels.** To test whether HENA can produce *in vivo* vasodilation, we used a closed cranial window on anesthetized rats. Our technique allows visual *in vivo* monitoring of pial arterioles (50-100  $\mu$ m external diameter) that arise from major cerebral (including middle) arteries and are the principal vascular conduit for maintaining continuous blood flow to the brain (Baumbach and Heistad, 1985). Control (DMSO-containing) and HENA- or LCA-containing solutions were infused into the cerebral circulation *via* catheter in the carotid artery. As observed with control perfusion of pressurized cerebral arteries *in vitro*, infusion of vehicle-containing Sodium Saline Solution (SSS, 1 mL) caused a mild ( $\leq$ 5 %) and transient arteriolar dilation, which fully disappeared within 4 minutes after infusion (**Fig. 10A**). In contrast, 45  $\mu$ M of LCA or HENA rendered up to a  $\geq$ 10% additional dilation (i.e., on top of that evoked by vehicle). LCA- and HENA-induced dilation was sustained throughout 10 minutes of diameter monitoring after continuous drug infusion. Notably, HENA-induced arteriolar dilation was significantly

larger than that evoked by LCA during the 4<sup>th</sup> through the 10<sup>th</sup> minute after drug infusion (**Fig. 10A**). Moreover, the effect of each compound was reversible, gradually diminishing until fully gone within 5 minutes of washout by infusion of 1 mL of drug-free SSS. Finally, evaluation of systemic blood pressure by catheterization of the femoral artery indicated that intracarotid infusion of HENA failed to modify mean arterial blood pressure:  $71 \pm 2.5$  vs.  $69 \pm 4.1$  mmHg before HENA intracarotid infusion and during 4-6<sup>th</sup> minute following HENA intracarotid infusion, respectively. This result indicates that pial dilation in response to intracarotid HENA is not related to, or, at least, is not largely caused by, a fall in systemic blood pressure. Rather, HENA-induced cerebral artery dilation observed *in vivo* during and immediately after HENA intracarotid infusion represents a true dilator response to HENA of the cerebral circulation.

To determine the involvement of BK channels in HENA-induced pial arteriolar dilation, we used the selective BK channel blocker paxilline, which was topically applied at 1  $\mu$ M on the brain surface. Paxilline application caused robust constriction of arterioles, with up to 15% reduction in diameter from pre-paxilline values (**Fig. 10B**). This result underscores the key role of BK channels in limiting vessel constriction. When compared to control (DMSO-containing SSS), carotid artery infusion of 45  $\mu$ M HENA in presence of paxilline failed to cause any change in arteriolar diameter throughout 10 minutes of continuous diameter monitoring (**Fig. 10D**). This lack of response to HENA infusion in the presence of paxilline points at BK channels as molecular targets of HENA-driven pial arteriolar *in vivo* dilation. Finally, washout of paxilline from the brain surface caused a rebound increase in arterial diameter back to pre-paxilline levels (**Fig. 10B**), documenting arteriole viability after the several experimental manipulations.

Besides BK, cerebrovascular smooth muscle tone is also controlled by purely voltage-gated K<sup>+</sup> (K<sub>v</sub>) channels (Faraci and Sobey, 1998). To determine the selectivity of BK channel

involvement in HENA-induced dilation, we evaluated HENA action in the presence of 4-aminopyridine (4-AP). At submillimolar to low millimolar concentrations, AP blocks most  $K_v$  but not BK channels in rat cerebral arteries (Liu et al., 2004). Topical application of 0.8 mM 4-AP on the surface of the brain caused an immediate decrease in diameter up to 15% from pre-4-AP level (**Fig. 10C**). In contrast to data obtained in presence of HENA+paxilline, carotid infusion of HENA in presence of 4-AP caused arteriolar dilation which was significantly higher compared to infusion of vehicle-containing SSS (**Fig. 10D**). Moreover, HENA-induced dilation reached 10% on top of control value, which was identical to HENA-induced dilation determined in absence of 4-AP (**Figs. 10A, D**). These results indicate that  $K_v$  channels other than BK do not play a major role in HENA-induced *in vivo* dilation of pial arterioles. Rather, the HENA effect is attributed to selective targeting of BK channels by the nonsteroidal ligand. Finally, washout of 4-AP from the brain surface caused a rebound increase in artery diameter back to pre-4-AP levels (**Fig. 10C**), documenting arteriole viability after the several experimental manipulations.

## Discussion

It has been consistently documented that BK auxiliary subunits of type I are particularly abundant in smooth muscle in general and arterial myocytes in particular (Orio et al., 2002; Brenner et al., 2000a). Moreover, this subunit plays a central role in promoting smooth muscle relaxation and vasodilation (Brenner et al., 2000b). These previous findings raised hope that selective targeting of BK  $\beta$ 1 subunits by small agents could be an effective mechanism to evoke vasodilation while barely targeting BK channels in tissues other than smooth muscle or other receptors. Present data unveil for the first time that HENA, an exogenous and nonsteroidal agent, evokes a robust dilation of resistance size, cerebral arteries both *in vitro* and *in vivo* by smooth muscle BK channel activation. The strong quantitative similarities between HENA *in vitro* dilation of isolated, de-endothelized arteries and the agent-induced dilation of cerebral arteries when administered *via* carotid artery to the intact animal strongly suggest that neither circulating nor endothelial factors exert a major regulatory effect on HENA targeting of smooth muscle BK channels. Moreover, this action involves HENA recognition by the recently discovered steroid-sensing site present in the BK  $\beta$ 1 protein (Bukiya et al., 2011). Therefore, our study demonstrates that LCA and related steroids can be used as a template for the search, design and development of nonsteroidal vasodilators that selectively target  $\beta$ 1-containing BK channels.

Before our discovery of HENA, a few ligands have been claimed to require the presence of BK  $\beta$ 1 subunits to increase BK channel activity. Among physiological, endogenous ligands,  $17\beta$ -estradiol at low  $\mu$ M levels has been reported to increase the activity of recombinant BK constructs made of hsl $\alpha$  (from human myometrium)+ $\beta$ 1 subunits while failing to activate homomeric hsl $\alpha$  channels. This study also showed [ $^3$ H]  $17\beta$ -estradiol specific binding and bovine serum albumin-bound fluorescent  $17\beta$ -estradiol labeling in membranes expressing BK  $\alpha$

and  $\beta 1$  subunits but not in membranes expressing solely  $\alpha$  subunits (Valverde et al., 1999). A later study, however, demonstrated that sub- $\mu\text{M}$  levels of  $17\beta$ -estradiol modulated BK activity in absence of BK  $\beta 1$  (Korovkina et al., 2004). Also regarding physiologically-relevant steroids, the vasoactive properties of LCA and other bile acids have been known for several decades (Bomzon and Ljubuncic, 1995), and BK channel activation by LCA and related cholanes has been demonstrated in both vascular and nonvascular smooth muscle (Dopico et al., 2002; Bukiya et al., 2007). In particular, our group has demonstrated that myocyte BK channel activation by LCA renders cerebral artery dilation, a steroid action that requires selective ligand recognition by a steroid-recognition site identified in the BK  $\beta 1$  TM2 (Bukiya et al., 2007; 2011). Due to their steroidal character, LCA and related cholanes exert numerous biological effects within and outside the vascular bed involving interactions with multiple membrane and cytosolic proteins, and transcription factors (Makishima et al., 2002; Maruyama et al., 2002; Gerasimenko et al., 2006; Modica and Moschetta, 2006). As found for tauro lithocholic acid 3-sulfate in pancreatic acinar cells (Gerasimenko et al., 2006), we showed here that  $\mu\text{M}$  LCA raised overall cytosolic  $\text{Ca}^{2+}_i$  in endothelium-free cerebral artery, an effect not observed with HENA (**Supplemental Figure 3**). Thus, any possible use of LCA as vasodilator might require additional interventions to reduce bile acid-induced increase in  $\text{Ca}^{2+}_i$ , as this increase would oppose smooth muscle relaxation and vasodilation. More generally, widespread side effects secondary to cholane steroid-recognition by off-target proteins (i.e., other than  $\beta 1$ -containing BK channels) would severely limit the use of LCA and related cholanes as vasodilators in clinical practice. A similar limitation would likely apply to other physiological steroids; the targeting of multiple BK subunits and tissues by physiologically-relevant steroids is reviewed elsewhere (Dopico et al., 2012).

Regarding ligands exogenous to the animal organism, the glycoside dehydrosoyasaponin-I (DHS-I) derived from the medicinal herb *Desmodium adscendens*, has been shown to activate  $\beta 1$  subunit-containing BK channels at nM concentrations (McManus et al., 1993; 1995). Unfortunately, DHS-I is only effective in activating BK channels when applied from the intracellular side of the membrane (McManus et al., 1995), drastically limiting DHS-I delivery to myocyte BK channels *via* circulation. This limitation does not apply to HENA, as demonstrated by present data (**Figs. 8-10**).

Other known BK channel activators do not discriminate between the different types of BK  $\beta$  subunits or do not require them at all. For example, the voltage-sensitive oxonol dye and related analogs are able to activate  $\beta 1$ -containing and neuronally-abundant,  $\beta 4$ -containing BK channels (Morimoto et al., 2007). The benzimidazolone NS1619 (Papassotiriou et al., 2000), the benzofuroindole (CTBIC), and the tetrahydroquinoline termed compound Z (Lee et al., 2012; Ponte et al., 2012) all induce BK channel activation in absence of BK  $\beta 1$ , with drug action being likely mediated by the ubiquitously distributed BK  $\alpha$  subunit. It remains unknown whether NS1619 amido derivatives promote relaxation of KCl-precontracted isolated thoracic aortic rings *via* BK  $\beta 1$  subunits (Calderone et al., 2008). Likewise, it remains unknown whether NS11021 and related anthraquinones activate BK channels (Roy et al., 2012) *via* BK  $\beta 1$  and, if so, whether other BK  $\beta$ s can substitute for  $\beta 1$  in mediating drug action. Therefore, to our knowledge, HENA is the only known nonsteroidal BK channel activator and vasodilator that 1) can be delivered from the extracellular side of the membrane, and 2) selectively targets  $\beta 1$ -containing BK channels. From a basic research standpoint, HENA can be used as a pharmacological tool alternative to LCA for detecting  $\beta 1$ -containing BK channels and targeting the BK  $\beta 1$  subunit steroid-sensing site in biological preparations.

Consideration of HENA and related compounds in clinical settings, however, requires some additional discussion. HENA belongs to a large family of naturally-occurring pentacyclic triterpenes (PT) derived from herbal sources. Several derivatives having the PT skeleton are antitumoral agents and inhibitors of glycogen phosphorylase and  $\text{Na}^+/\text{K}^+$ -ATPase (Kaneda et al., 1992; Terasawa et al., 1992; Liang et al., 2011). Although a current CAPLUS database search on PTs results in over 100 reports, none of these reports points at vasoactive properties of PT-related compounds. Thus, HENA may possess unique chemical features that restrict its actions to the vascular smooth muscle and, in particular, BK channels. Indeed, our data show that HENA not only discriminates between BK  $\beta_1$  and other BK  $\beta$ s (**Fig. 5**), but also fails to functionally interact with smooth muscle Kv TM6 channels other than BK (**Fig. 10D**). These outcomes may be explained by the low degree of similarity between the HENA-sensing BK  $\beta_1$  subunit and other known proteins. Indeed, Thr169, shown here critical for HENA action, is unique to BK channels of the  $\beta_1$  type. The selectivity of HENA on BK  $\beta_1$  containing BK channels is critical from a drug development point of view, as a good drug development candidate should act on a minimal set of targets to evoke few side effects.

A fundamental feature that determines the clinical utility of a drug candidate is its efficacy on the tissue of interest. We show here that 45  $\mu\text{M}$  HENA whether applied *in vitro* to a de-endothelized artery or *in vivo* through intra-carotid infusion increases cerebral artery diameter by ~15% (**Fig. 10**). This change is expected to evoke a robust increase in cerebral blood flow (by 45%) (Gourley and Heistad, 1984), and would rescue cerebral protein synthesis and likely prevent the extracellular accumulation of glutamate and lactate that are found upon a 20-50% reduction of CBF (Hossmann, 1994).

The identification of the LCA-steroid site as mediating HENA action on BK channels, followed by further computational modeling and organic chemical synthesis would lead to the design of  $\beta 1$  subunit-containing BK channel antagonists that could be used to reduce HENA-induced vasodilation should this effect exceed a desired set-point. Therefore, our study raises the hypothesis that HENA is an attractive drug development candidate for treating conditions associated with cerebral artery constriction such as cerebral vasospasm and ischemia. Although HENA efficacy on cerebral artery diameter is impressive, its *in vitro* potency is not optimal ( $EC_{50}=45 \mu M$ ). Thus, further medicinal chemistry efforts are likely needed to optimize the HENA structure and yield more potent, while similarly effective, vasodilators without compromising selectivity towards the BK  $\beta 1$  subunit. In conclusion, our study has demonstrated that selective targeting of the steroid site in  $\beta 1$ -containing BK channels by a nonsteroidal, exogenous and novel ligand is an effective way to evoke vasodilation.

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### **Authorship contributions**

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*Performed data analysis:* Bukiya, McMillan

*Wrote or contributed to the writing of the manuscript:* Bukiya, McMillan, Patil, Leffler, Parrill,

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## Footnotes

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## Figure legends

### **Fig. 1. The non-steroid HENA shows substantial structural similarity with lithocholic acid**

**(LCA).** A. Chemical structures of LCA and HENA. In HENA, a zigzag line highlights the point of hydrolysis of methyl 3-hydroxyolean-12-en-30-oate to render HENA. B. Flexible alignment of LCA and HENA in their ionized forms reveals close similarity in the overall shape of the molecules and overlap in critical functional groups. Oxygen atoms are shown in grey.

### **Fig. 2. HENA is more efficient than LCA in increasing the activity of cbv1+β1 channels.**

Single-channel recordings from I/O patches excised from *X. oocytes* expressing cbv1+β1 channel complexes. Unitary current records depict BK channel activity before, during, and after 45 μM LCA (A) or 45 μM HENA application (B).  $V_m = -40\text{mV}$ ,  $[\text{Ca}^{2+}]_{\text{free}} = 10\ \mu\text{M}$ . Here and in all other figures: channel openings are shown as downward deflections; arrows indicate the baseline; control and washout correspond to vehicle-containing bath solution. C. HENA- and LCA-induced increase in BK channel NPo shows no correlation with the channel's basal (pre-drug) activity. D. Concentration response curves (CRCs) for HENA and LCA. Each point represents the average of no less than 3 patches, each patch obtained from a different oocyte. \*Different from LCA ( $P < 0.05$ ). E. Concentration-response curves where drug-induced BK channel activation at a given concentration is normalized to the drug's maximal effect show that HENA and LCA have similar  $\text{EC}_{50}$ .

### **Fig. 3. HENA activates BK channels within a wide voltage range.**

A. HENA (45 μM) increases macroscopic current mediated by cbv1+β1 subunits expressed in *X. laevis* I/O membrane patches. Voltage steps of 200 ms duration were applied from -150 to +150 mV in 10

mV increments from  $V_{\text{holding}}=0$  mV;  $[\text{Ca}^{2+}]_{\text{free}}=10$   $\mu\text{M}$ . B. G/Gmax-V curves from cbv1+ $\beta$ 1 channels at 10  $\mu\text{M}$   $[\text{Ca}^{2+}]_{\text{free}}$  show a parallel leftward shift in the presence of 45  $\mu\text{M}$  HENA when compared to control.  $V_{1/2}$  denotes the transmembrane voltage ( $V_m$ ) at which G/Gmax=0.5. Each data point represents the averaged from  $\geq 4$  patches.

**Fig. 4. HENA-induced BK channel activation is observed at physiologically relevant  $\text{Ca}^{2+}_i$  levels.**

A. Single channel records from cbv1+ $\beta$ 1 complexes expressed in *X. oocytes* show an increase in NPo in response to 45  $\mu\text{M}$  HENA.  $V_m=-40$  mV,  $[\text{Ca}^{2+}]_{\text{free}}=0.3$   $\mu\text{M}$ . B. Averaged responses of cbv1+ $\beta$ 1 channels to 45  $\mu\text{M}$  HENA, obtained at 0.3 and 10  $\mu\text{M}$   $[\text{Ca}^{2+}]_{\text{free}}$ . Each data point was obtained from no less than 4 patches; each patch obtained from a different cell. A horizontal dashed line defines lack of HENA effect. \*Different from pre-HENA NPo ( $P<0.05$ ).

**Fig. 5. BK  $\beta$ s 2-4 fail to substitute for  $\beta$ 1 in providing HENA sensitivity to the BK complex.**

A. Unitary current records obtained in I/O patches from *X. oocytes* that express BK channels of different subunit composition.  $V_m=-40$  mV,  $[\text{Ca}^{2+}]_{\text{free}}=10$   $\mu\text{M}$ . B. Average increase in BK activity (NPo) in the presence of 150  $\mu\text{M}$  HENA. Each point was obtained from no less than 4-5 patches. \*Different from cbv1 ( $P<0.05$ ).

**Fig. 6. Threonine 169 within BK  $\beta$ 1 TM2 confers HENA sensitivity to the BK complex.**

A. Channels resulting from cbv1 and engineered  $\beta$ 4 subunits containing the  $\beta$ 1 TM2 domain (cbv1+ $\beta$ 4TM21) retain sensitivity to 45  $\mu\text{M}$  HENA. B. Currents from cbv1+ $\beta$ 1T169A are resistant to HENA. In A and B:  $V_m=-40$  mV,  $[\text{Ca}^{2+}]_{\text{free}}=10$   $\mu\text{M}$ . C. Averaged responses to 45  $\mu\text{M}$  HENA from BK complexes containing modified  $\beta$ 1 subunits. HENA-resistant cbv1

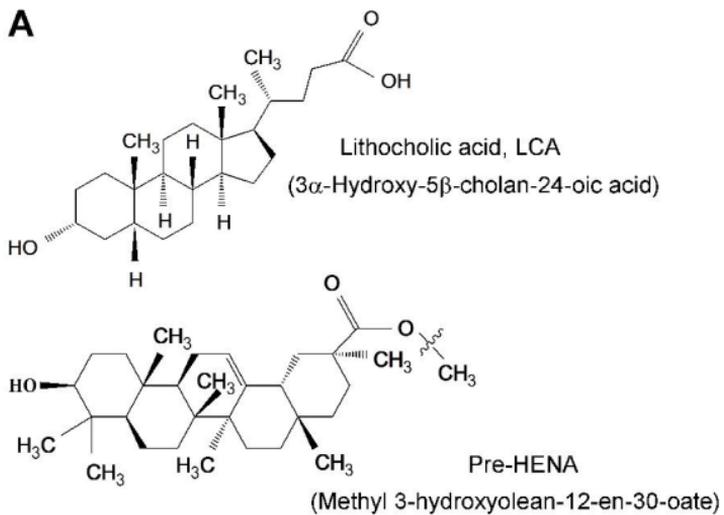
homomers and HENA-sensitive  $cbv1+\beta1$  heteromers serve as negative and positive controls, respectively. Each point was obtained from 4-5 patches. \*Different from  $cbv1$  ( $P<0.05$ ).

**Fig. 7. HENA increases the activity of rat cerebral artery myocyte BK channels more effectively than LCA.** A-B. Single BK channel recordings obtained in I/O patches excised from freshly isolated rat cerebral artery myocytes. Records depict native BK channel activity before, during, and after 45  $\mu\text{M}$  LCA (A) or 45  $\mu\text{M}$  HENA application (B).  $V_m=-40\text{mV}$ ,  $[\text{Ca}^{2+}]_{\text{free}}=3\ \mu\text{M}$ . C. CRCs for HENA and LCA. Each point represents the average of no less than 3 patches, each patch excised from a different oocyte. \*Different from LCA ( $P<0.05$ ). D. Concentration-response curves where drug-induced BK channel activation at a given concentration is normalized to the drug's maximal effect show that HENA and LCA have similar  $\text{EC}_{50}$ .

**Fig. 8. HENA dilates pressurized cerebral arteries *via* activation of BK channels and independently of an intact endothelium.** A. Diameter trace from de-endothelized, pressurized cerebral artery. After development of myogenic tone, acute application of 45  $\mu\text{M}$  HENA causes reversible dilation, which is larger and lasts longer than dilation by 45  $\mu\text{M}$  LCA. B. CRCs for HENA and LCA on artery diameter. Each point represents the average of  $\geq 3$  arteries, each artery harvested from a different rat. \*Different from LCA ( $P<0.05$ ). C. Artery diameter traces showing that HENA action is ablated by selective BK channel block with 1  $\mu\text{M}$  paxilline. D. Average change in artery diameter evoked by 45  $\mu\text{M}$  HENA in absence ( $n=3$ ) and presence ( $n=3$ ) of 1  $\mu\text{M}$  paxilline. \*\*\*Different from HENA-induced dilation in the absence of paxilline ( $P<0.001$ ).

**Fig. 9. HENA fails to dilate pressurized arteries from BK  $\beta$ 1 subunit-lacking (*KCNMB1* K/O) mice.** Cerebral artery diameter traces show that acute application of 45  $\mu$ M HENA causes sustained diameter increase (dilation) in arteries from BK  $\beta$ 1 subunit-containing C57BL/6 mouse (A) but not in arteries from *KCNMB1* K/O mouse (B). C. Average diameter data in response to HENA (dark bars) (n=4) compared to pre-HENA levels (hollow bars). \*Different from diameter before HENA application (P<0.05).

**Fig. 10. HENA dilates pial arterioles *in vivo* through selective activation of BK channels.** A. Pial arteriole dilation upon LCA or HENA infusion as function of time. Insert: snapshot from the surface of the brain *via* a cranial window depicting computer detection of arteriole external diameter before application of 45  $\mu$ M LCA or HENA infusion *via* the carotid artery. B. Averaged pial arteriolar diameter (as % of diameter before channel blocker) in response to topical application of the selective BK channel blocker paxilline (1  $\mu$ M) on the brain surface. C. Averaged pial arteriolar diameter (as % of diameter before blocker) in response to topical application of 4-aminopyridine (0.8 mM), which blocks Kv channels other than BK. In panels B and C, each data point represents averaged from no less than 3 animals; a horizontal dashed line defines arteriolar diameter before blocker application; \*Different from pre-blocker level (P<0.05). D. Pial arteriole dilation upon HENA infusion in presence of paxilline or 4-AP as function of time. \*Different from diameter upon vehicle-containing bath solution infusion (P<0.05); #Different from LCA-induced pial arteriolar dilation (P<0.05).



**Figure 1**

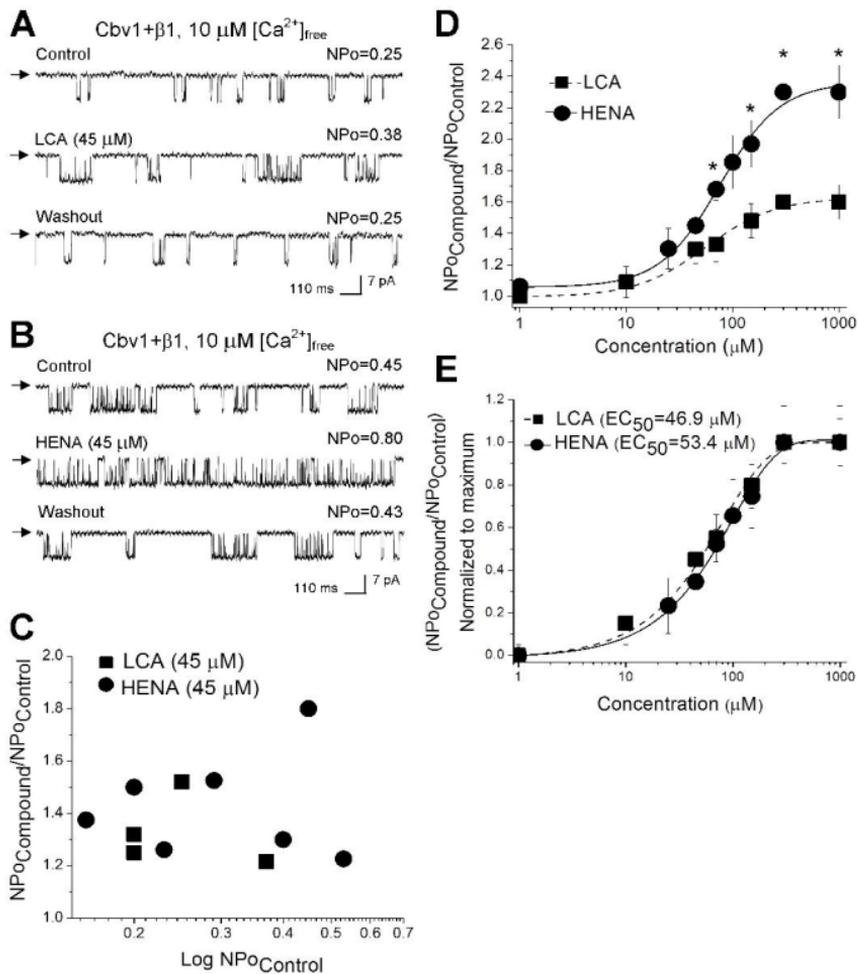
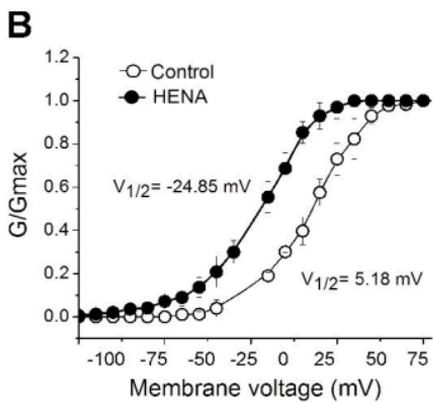
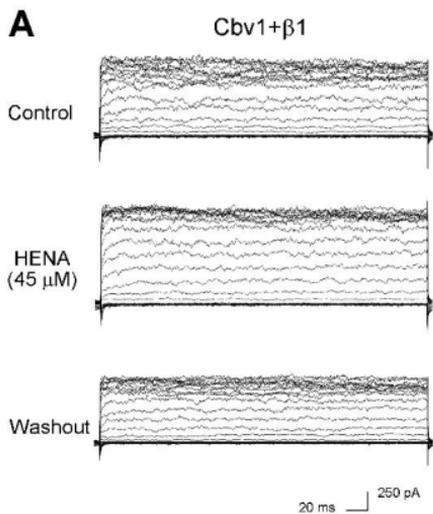
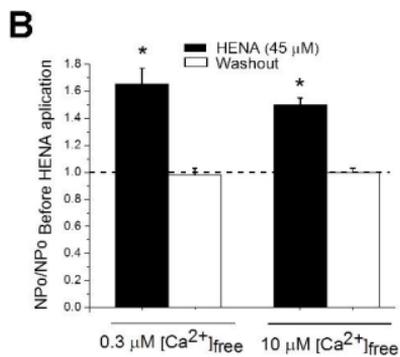
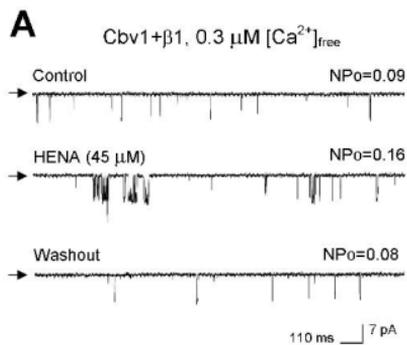


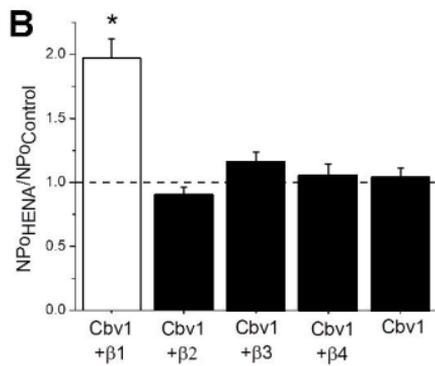
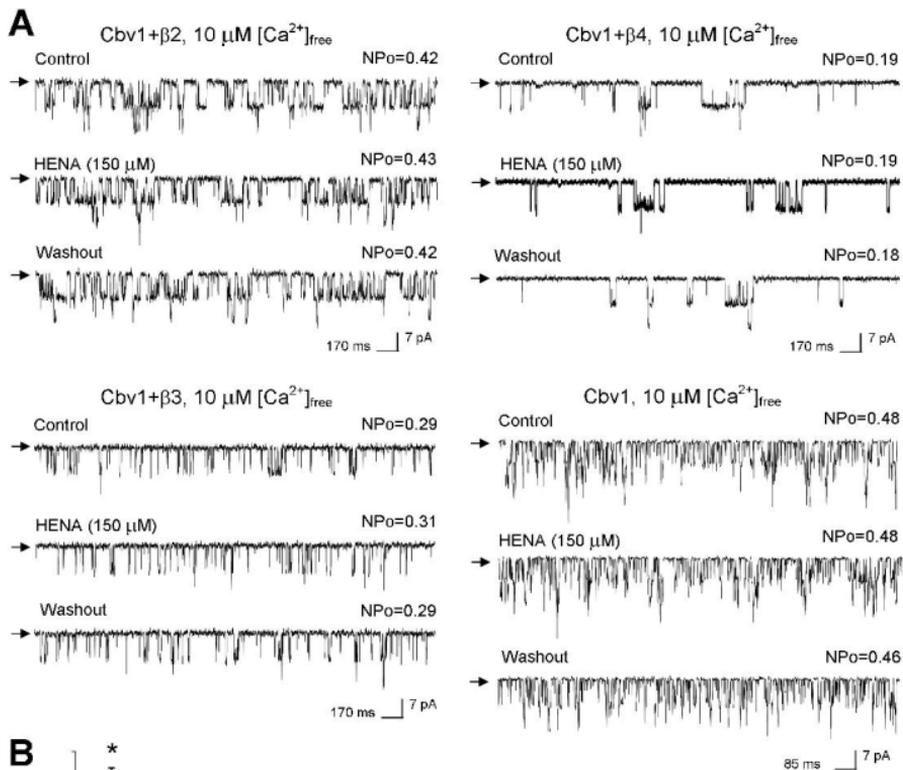
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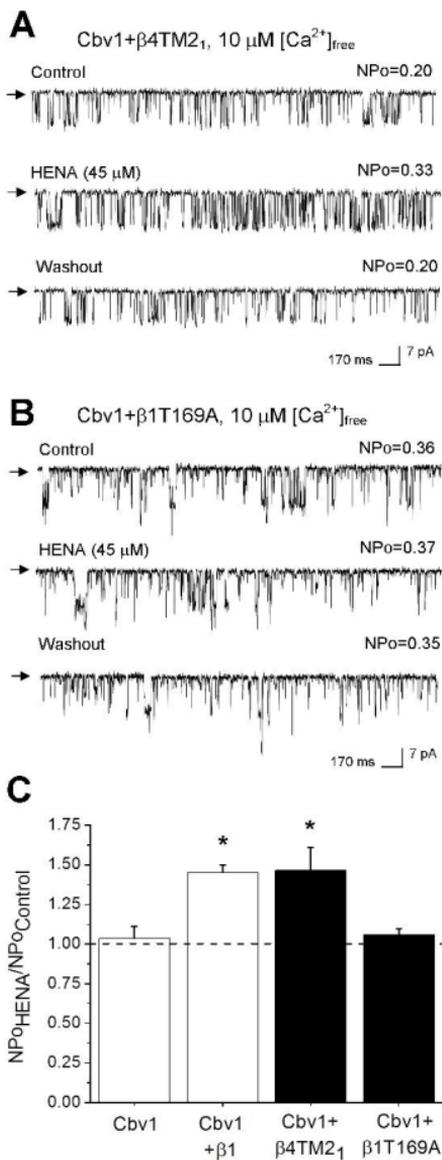
**Figure 3**



**Figure 4**



**Figure 5**



**Figure 6**

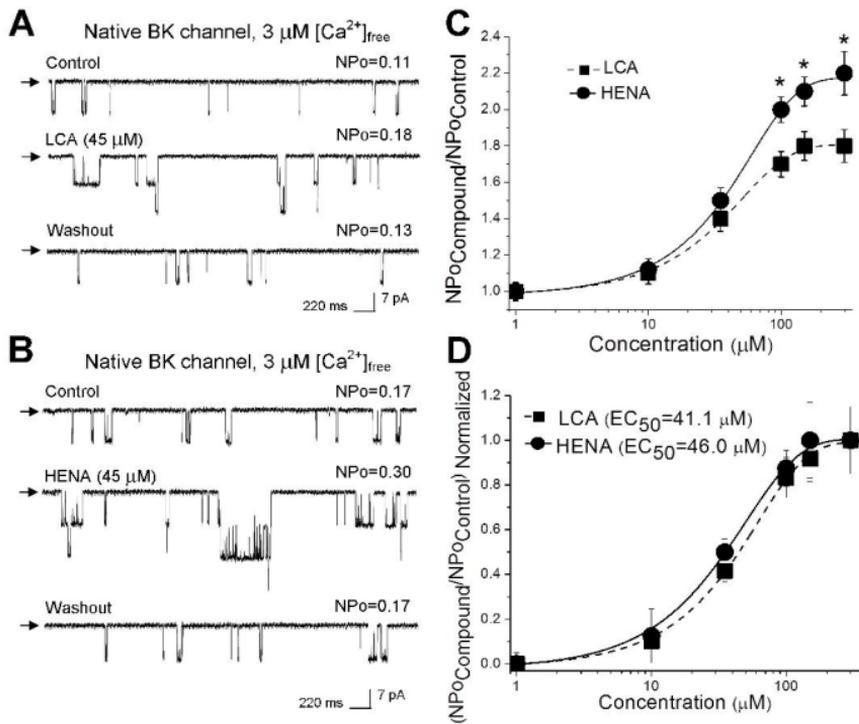


Figure 7

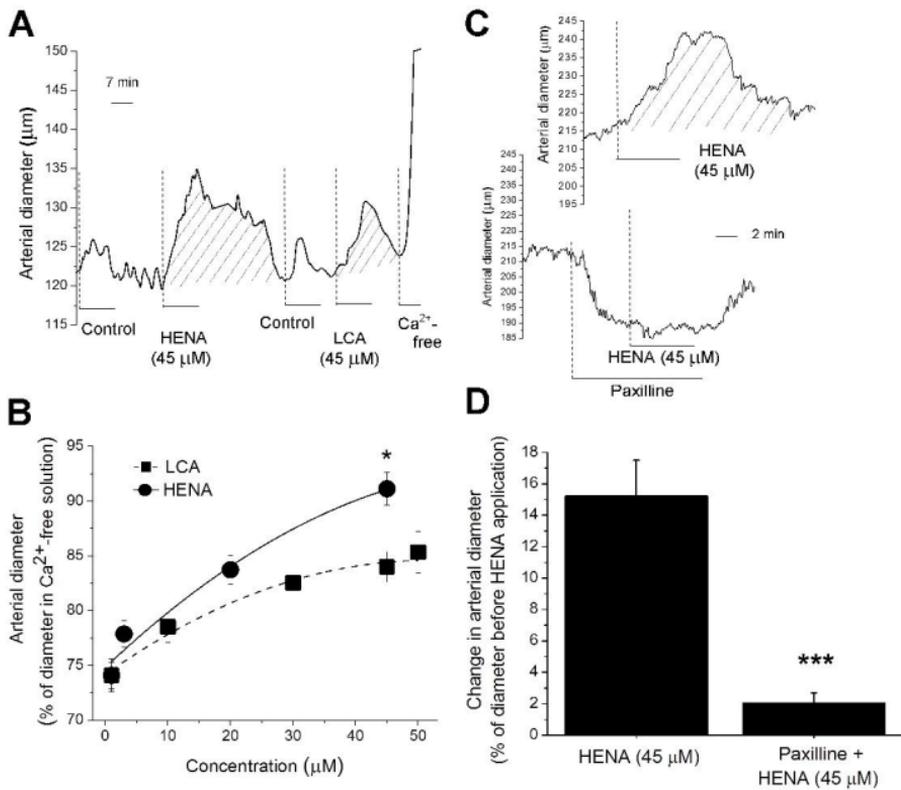


Figure 8

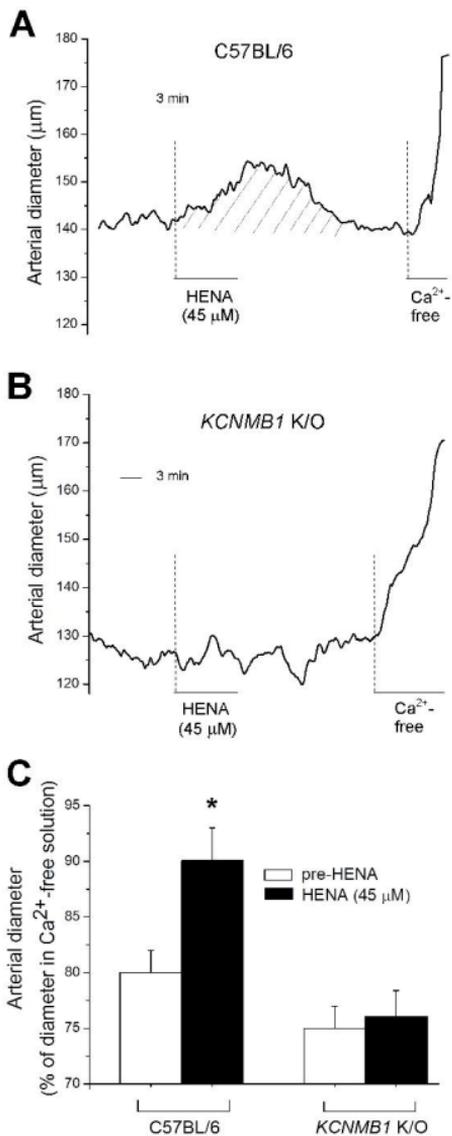


Figure 9

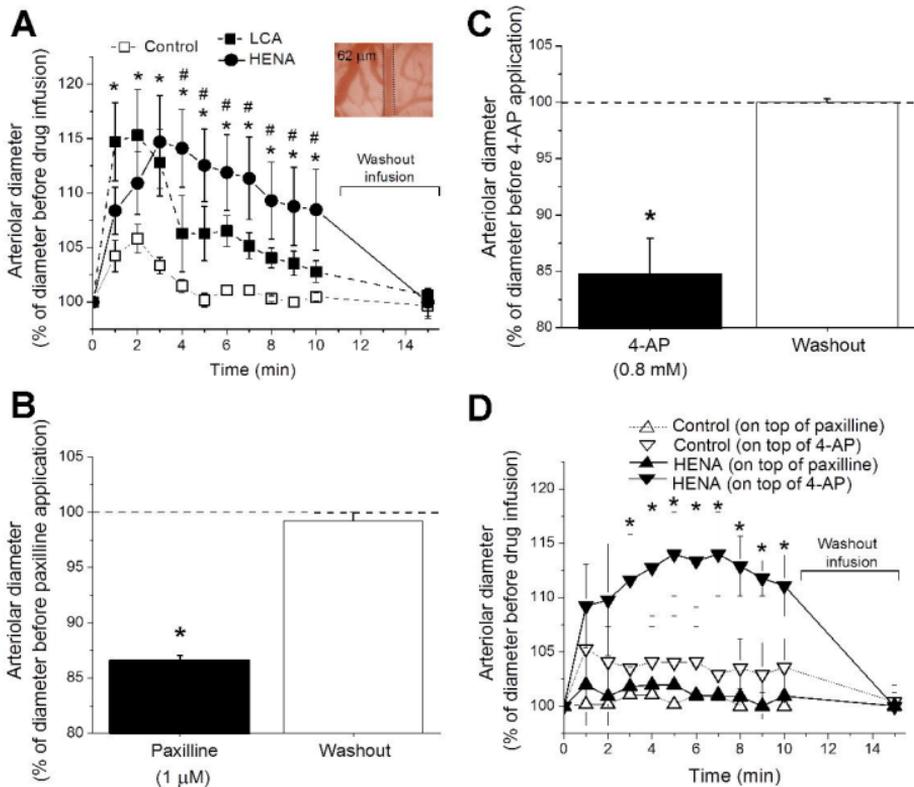
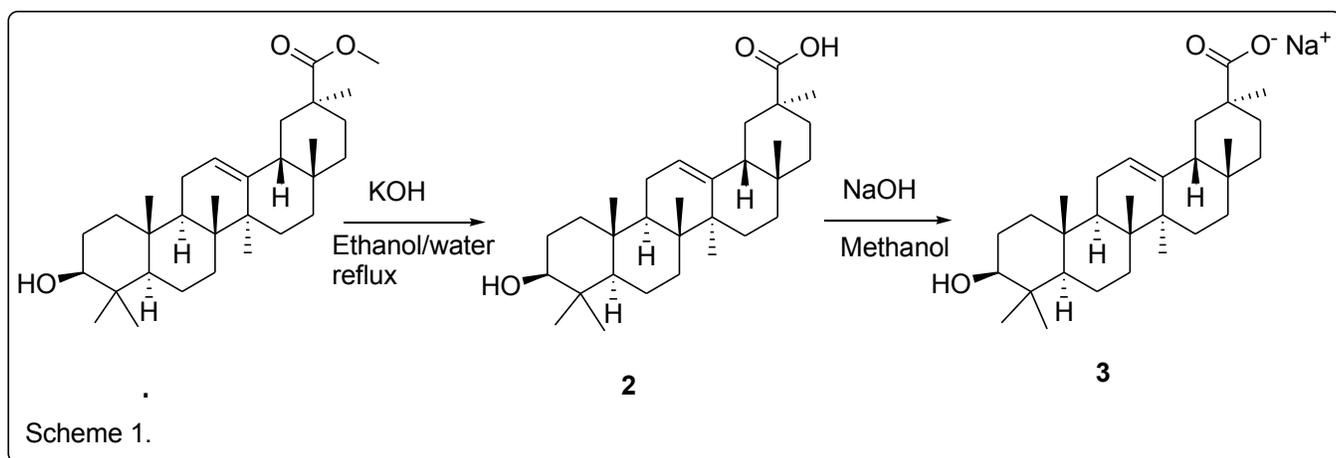
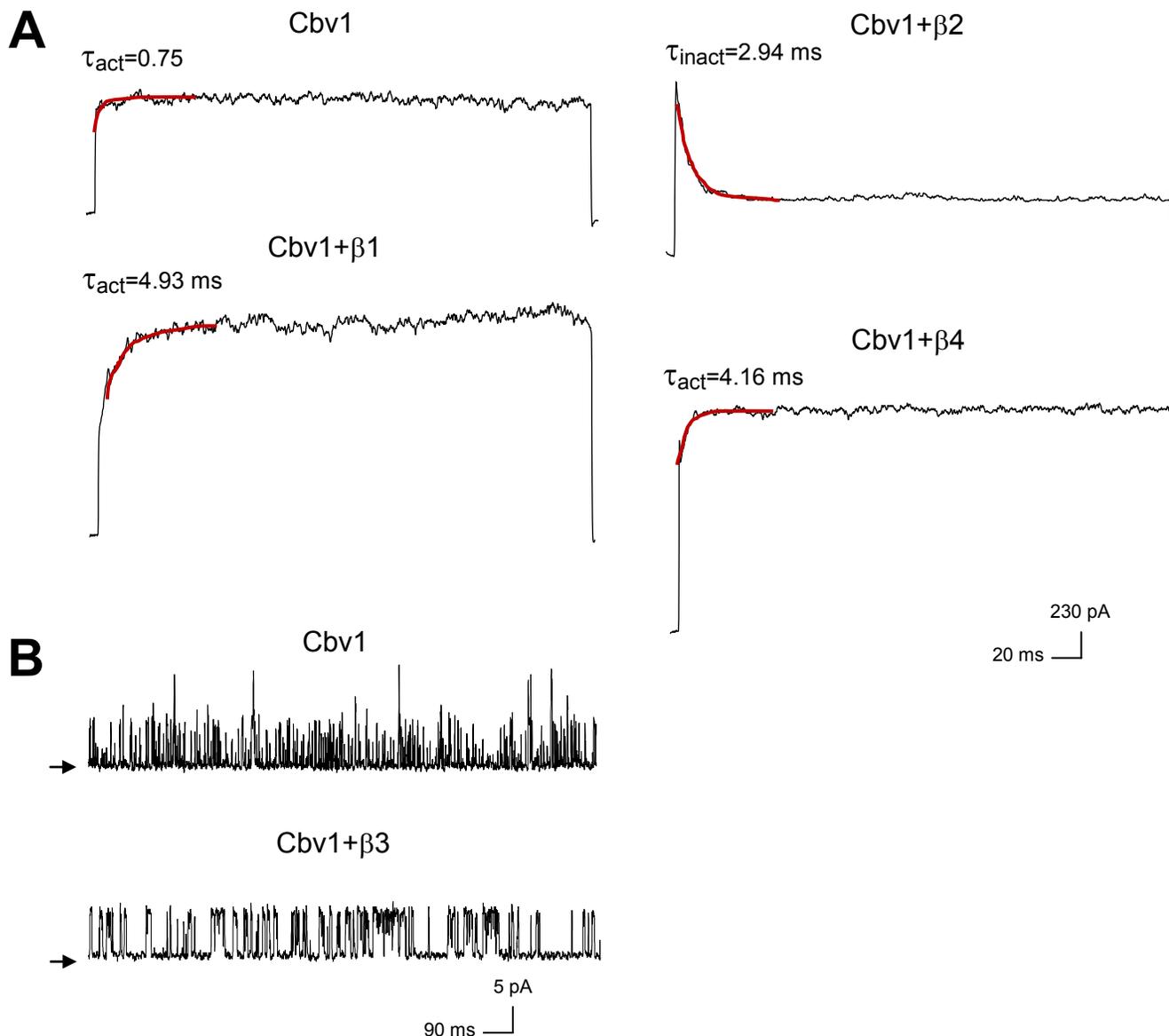


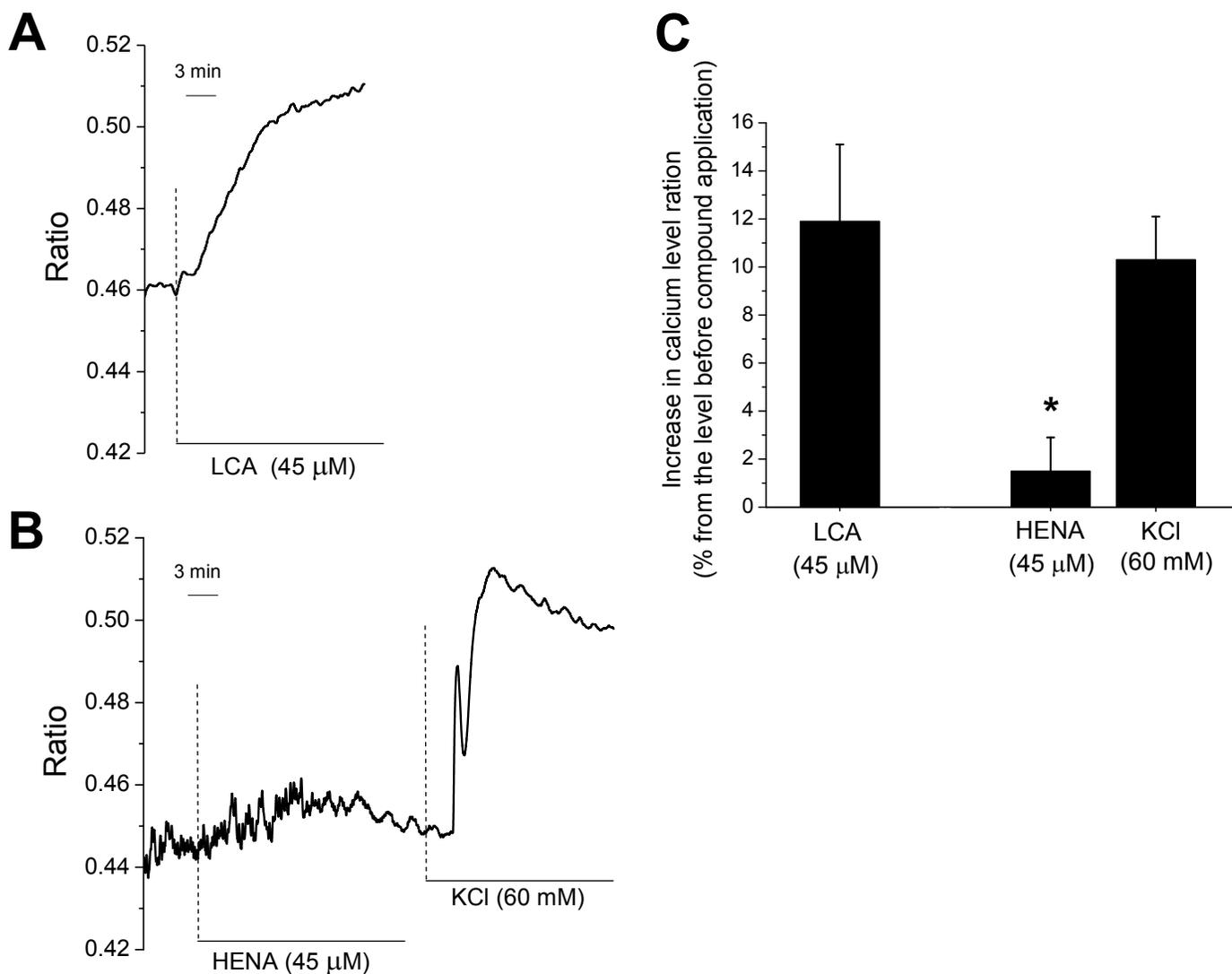
Figure 10



**Suppl. Fig. 1. Two-step chemical conversion of 3'-hydroxy-olean-12-en-29-oic acid methyl ester into “HENA”.** 3'-hydroxy-olean-12-en-29-oic acid methyl ester ( compound 1) was hydrolyzed to obtain 3'-hydroxy-olean-12-en-29-oic acid (compound 2). Purified acid (2) was further converted to the corresponding sodium salt (compound 3, “HENA”).



**Suppl. Fig. 2. Phenotypic characterization of macroscopic and single-channel currents evoked by  $cbv1+\beta(1-4)$  subunit expression in *Xenopus laevis* oocytes.** A. Representative current records from I/O macropatches expressing different BK channel subunit combinations ( $cbv1\pm\beta 1$ ,  $\beta 2$ , or  $\beta 4$ ). Macroscopic currents were evoked by 200 ms-long, 10 mV depolarizing steps from  $-150$  to  $+70$  mV from a holding potential= $0$  mV. Red fitting lines underscore the different current activation/inactivation kinetics for each channel subunit combination. B. Original records of single BK channel activity showing that expression of  $cbv1+\beta 3$  heteromers results in longer channel openings when compared to those from homomeric  $cbv1$ .  $V_m=+40$ mV, free  $[Ca^{2+}]_i=10$   $\mu$ M.



**Suppl. Fig. 3. Ratiometric detection of changes in arterial wall  $[Ca^{2+}]_i$  in presence of LCA vs. HENA.**

A. Original trace showing LCA-induced increase in  $[Ca^{2+}]_i$ . B. Original trace showing lack of  $[Ca^{2+}]_i$  increase by HENA, with high KCl being used as positive control. C. Averaged changes in arterial wall  $[Ca^{2+}]_i$  evoked by LCA, HENA, and KCl. \*Different from LCA-induced increase in arterial wall  $[Ca^{2+}]_i$  ( $P < 0.05$ ). In A-C, data were obtained in the presence of BK channel selective blockade by 1  $\mu$ M paxilline.

“Cerebrovascular dilation via selective targeting of the cholane steroid-recognition site in the BK channel beta1 subunit by a novel nonsteroidal agent”, Bukiya A, McMillan J, Fedinec A, Patil S, Miller D, Leffler C, Parrill A, Dopico A. Molecular Pharmacology.

### Supplemental Methods

**Arterial wall  $[Ca^{2+}]_i$  measurements.** Adult male Sprague-Dawley rats were decapitated using a guillotine. Middle cerebral arteries were isolated on ice under microscope (Nikon SMZ645; Nikon, Tokyo, Japan) and cut into 1 to 2 mm-long segments. Segments were incubated in physiological saline solution (composition in main text) containing 3  $\mu$ M fura-2AM for 45 min. Endothelium was removed by passing an air bubble into the vessel lumen for 90 seconds prior to vessel cannulation. A segment was cannulated at each end, bathed by physiological saline (PSS, composition in main text) containing 1  $\mu$ M paxilline to block BK channels, and kept in the dark for 15 min. Fura-2 was alternatively excited at 340 or 380 nm by using a PC-driven hyper-switch (Ionoptix, Milton, MA) for ratiometric detection of free  $[Ca^{2+}]$  levels within each arterial segment.