Cerebrovascular Dilation via Selective Targeting of the Cholane Steroid-Recognition Site in the BK Channel β1-Subunit by a Novel Nonsteroidal Agent

Anna N. Bukiya, Jacob E. McMillan, Alexander L. Fedinec, Shivaputra A. Patil, Duane D. Miller, Charles W. Leffler, Abby L. Parrill, and Alex M. Dopico

Departments of Pharmacology (A.N.B., A.M.D.), Physiology (A.L.F., C.W.L.), and Pharmaceutical Sciences (S.A.P., D.D.M.), University of Tennessee Health Science Center, and Department of Chemistry and Computational Research on Materials Institute (CROMIUM), University of Memphis, Memphis, Tennessee (J.E.M., A.L.P.)

Received November 7, 2012; accepted March 1, 2013

ABSTRACT

The Ca2+/voltage-gated K+ large conductance (BK) channel β1 subunit is particularly abundant in vascular smooth muscle. By determining their phenotype, BK β1 allows the BK channels to reduce myogenic tone, facilitating vasodilatation. The endogenous steroid lithocholic acid (LCA) dilates cerebral arteries via BK channel activation, which requires recognition by a BK β1 site that includes Thr169. Whether exogenous nonsteroidal agents can access this site to selectively activate β1-containing BK channels and evoke vasodilatation remain unknown. We performed a chemical structure database similarity search using LCA as a template, along with a two-step reaction to generate sodium 3-hydroxyolean-12-en-30-oate (HENA). HENA activated the BK (cbv1 + β1) channels cloned from rat cerebral artery myocytes with a potency (EC50 = 53 μM) similar to and an efficacy (×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 the channels made of cbv1 + β2, β3, β4, or β1T169A, indicating that this drug selectively targets β1-containing BK channels via the BK β1 steroid-sensing site. HENA (3–45 μM) dilated the rat and C57BL/6 mouse pressurized cerebral arteries. Consistent with the electrophysiologic results, this effect was larger than that of LCA. HENA failed to dilate the arteries from the KCNM1 knockout mouse, underscoring BK β1’s role in HENA action. Finally, carotid artery-infusion of HENA (45 μM) dilated the pial cerebral arteries via selective BK-channel targeting. In conclusion, we have identified for the first time a nonsteroidal agent that selectively activates β1-containing BK channels by targeting the steroid-sensing site in BK β1, rendering 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 Ca2+-gated K+ (BK) channels result from the association of four identical α (slo1) subunits, which are ubiquitously distributed (Ghata et al., 2006; Salkoff et al., 2006). In most tissues, however, a homotetramers are accompanied by small, two transmembrane (TM2)-spanning accessory (β1–4) subunits that are encoded by four separate genes: KCNM1–4 (Orio et al., 2002). Remarkably, KCNM1 expression is highly tissue-specific, being abundant in the smooth muscle, including arterial myocytes (Brenner et al., 2000a,b; Orio et al., 2002). BK channels are critical determinants of artery myogenic tone: upon activation, they generate outward currents that counteract depolarization-induced Ca2+ entry, limiting constriction

1

1030

Copyright 2013 by The American Society for Pharmacology and Experimental Therapeutics

Molecular Pharmacology

1521-0111/03/5/1030–1044$25.00

http://dx.doi.org/10.1124/mol.112.083519

Mol Pharmacol 83:1030–1044, May 2013

This article has supplemental material available at molpharm.aspetjournals.org.

This work was supported by the National Institutes of Health National Heart, Lung and Blood Institute [Grants AA011560, HL104631, HL34059, and HL08269].

Preliminary data were previously presented as a poster presentation: Bukiya A, McMillan J, Fedinec A, Leffler C, Parrill A, Dopico A (2012) Sodium 3-hydroxyolean-12-en-30-oate is a novel and selective activator of β1 subunit-containing BK channels cloned from rat cerebral artery myocytes. HENA (3–45 μM) dilated the rat and C57BL/6 mouse pressurized cerebral arteries. Consistent with the electrophysiologic results, this effect was larger than that of LCA. HENA failed to dilate the arteries from the KCNM1 knockout mouse, underscoring BK β1’s role in HENA action. Finally, carotid artery-infusion of HENA (45 μM) dilated the pial cerebral arteries via selective BK-channel targeting. In conclusion, we have identified for the first time a nonsteroidal agent that selectively activates β1-containing BK channels by targeting the steroid-sensing site in BK β1, rendering vasodilation.

ABBREVIATIONS: aCSF, artificial cerebral spinal fluid; 4-AP, 4-aminopyridine; BK, large conductance; Ca2+/voltage-gated K+ compound 1, 3'-hydroxyolean-12-en-29-oic acid methyl ester; compound 2, 3'-hydroxyolean-12-en-29-oic acid; compound 3, sodium salt of 3'-hydroxyolean-12-en-29-oic acid; compound Z, (3aR,4S,9bS)-4-(Naphthalen-1-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-8-carboxylic acid; CTBIC, 4-chloro-7-(trifluoromethyl)-10H-benzo[3,2-b]indole-1-carboxylic acid; DHS-I, dehydrosoyasaponin-I; DM, dissociation medium; DMSO, dimethyl sulfoxide; HENA, sodium 3-hydroxyolean-12-en-30-oate; I/O, inside-out; K/O, knockout; KV, voltage-gated K+; LCA, lithocholic acid; N, number of functional channels in the membrane patch; NS1819, 4-(trifluoromethyl)-2-[[5-(trifluoromethyl)-2,3-dihydrobenzimidazol-1-yl]phenol; PAD, pial arteriolar diameter; Po, channel open probability; PSS, physiologic saline solution; PT, pentacryl triterpenes; SSS, sodium saline solution; TM, transmembrane; WT, wild type.
and favoring artery dilation (Jaggar et al., 1998; Ghatta et al., 2006). BK β1 subunits increase the apparent Ca$^{2+}$-sensitivity of the arterial smooth muscle BK channel, so this channel may be activated at voltage and Ca$^{2+}$ levels reached in contracting myocytes, thus exerting its negative feedback on myocyte contraction (Jaggar et al., 1998; Rusch, 2009).

The critical role of BK β1 in controlling myogenic tone and vasomotion has been underscored by several basic research and clinical findings. For example, KCNMB1 K/O (knockout) mice are characterized by uncoupling between vasodilating, RyR-generated Ca$^{2+}$-sparks and BK channel-generated spontaneous transient outward currents, 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, downregulation of BK β1 by NFATc3 activation contributes to systemic hypertension (Nieves-Cintrón et al., 2007). In humans, the gain-of-function Glu65Lys substitution in BK β1 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 β1 in limiting vascular smooth muscle contraction, its scarce expression in tissues other than smooth muscle, and its poor identity with other membrane proteins have 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 vasconstriction underlies numerous pathologic conditions, including cerebral vasospasm, ischemia after aneurysmal subarachnoid hemorrhage (Jordan and Nyquist, 2010), the reversible cerebral vasconstriction syndrome associated with nonaneurysmal subarachnoid hemorrhage, pregnancy, or exposure to certain drugs (Sattar et al., 2010), posttraumatic cerebral vasospasm after traumatic brain injury (Shahlaei et al., 2009), and abrupt-onset severe headaches (Ju and Schwedt, 2010). In addition, cerebral vasconstriction 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 choline steroids has been reported to activate smooth muscle BK channels (Dopico et al., 2002; Bukiya et al., 2007). Moreover, the most effective, lithocholic acid (LCA), evokes a robust cerebral artery in vitro dilation via activation of β1 subunit-containing BK channels (Bukiya et al., 2007). Recently, the LCA-recognition site was mapped to the BK β1 TM2 region. This steroid-recognition site includes Thr169 (Bukiya et al., 2011), a residue that is unique to BK β of type 1 (Brenner et al., 2000a), which likely explains why BK β2–4 fail to substitute for β1 in providing LCA sensitivity to BK channels (Bukiya et al., 2009a). Owing to their steroidal nature, LCA and related cholanes exert numerous biologic 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 inositol 1,4,5-trisphosphate receptors (Gerasimenko et al., 2006), cytosolic steroid-binding proteins, membrane transporters, and transcription factors (Modica and Moschetta, 2006). The resulting varied pharmacologic 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 β1 subunit-containing BK channels and, thus, effective vasodilators. We have tested and supported this hypothesis by combining a chemical database similarity search with 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, the latter 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 institution accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

#### Database Search and Computational Studies

A similarity search based on the LCA structure was performed throughout the Hit2Lead (https://www.hit2lead.com) database using a 70% similarity threshold. This screening rendered methyl 3-hydroxyolean-12-en-12,29-oic acid methyl ester (compound 1) to obtain 3′-hydroxy-olean-12-en-29-oic acid (compound 2) (Supplemental Fig. 1). Purified acid (2) was further converted to the corresponding sodium salt of 3′-hydroxy-olean-12-en-29-oic acid (compound 3) to improve solubility for probing of biologic activities.

#### Experimental Description

We purchased compound 1 from Hit2Lead.com (ChemBridge Corporation, San Diego, CA) for the hydrolysis, and used it without further purification. The organic solvents used for reaction as well as purification were purchased from Sigma-Aldrich (St. Louis, MO) and were used without further purification. Characterization of compound 2 was conducted using proton [1H] and [13C] 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 Compound 2 (2S,4aS,6aS,6bR,8aR,10S,12aR,12bR,14bR)-10-Hydroxy-2,4a,6a,6b,9,12a-Heptamethyloctacosane-2,3,4,5,6,11a,12a,12b,13,14b-Icosahydrolicene-2-Carboxylic Acid.

Compound 1 (0.085 mM, 40 mg) was dissolved in water throughout the Hit2Lead (https://www.hit2lead.com) database using a 70% similarity threshold. This screening rendered methyl 3-hydroxyolean-12-en-12,29-oic acid (compound 2) (Supplemental Fig. 1). Purified acid (2) was further converted to the corresponding sodium salt of 3′-hydroxy-olean-12-en-29-oic acid (compound 3) to improve solubility for probing of biologic activities.
mixture was extracted with chloroform. The chloroform layer was dried over anhydrous Na2SO4, then the chloroform was removed under reduced pressure. The crude material was purified by flash chromatography (ethyl acetate/hexanes; 1:9).

**Yield 34%;** $^1$H NMR (CDCl₃). Compound 2 was obtained in 34% yield, its structure being confirmed by $^1$H NMR (CDCl₃): δ 5.32 (t, J = 10.0 Hz, 1H), 3.25 (m, 1H, 3H-OCH), 1.78–2.05 (m, 6H), 1.72–1.48 (m, 7H), 1.46–1.13 (m, 14H, 2CH₃ groups were merged in the multiplet), 1.08–0.93 (m, 10H, 3CH₂ groups were merged in the multiplet), 0.90–0.72 (m, 8H, 2CH₃ groups were merged in the multiplet); $^{13}$C NMR (CDCl₃): δ 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, 27.61, 26.76, 25.64, 25.43, 22.95, 17.84, 16.26, 15.01, 14.93. Compound 2 molecular weight was determined by mass spectrometry (electrospray ionization): m/z (mass-to-charge ratio) 455.1 [M–H]⁺; Anal. Calcd. (C₂₉H₄₆O₃).

**Preparation of Compound 3.** NaOH (1.93 mg) was moistened with a drop of water in a small vial. To this vial, 0.5 ml of methanol and 11 mg of pure acid (2) were added and stirred for 5 minutes. The methanol was evaporated under reduced pressure, and then the residue was finally dried over a high vacuum. The residue was finally dried under a vacuum. The residue was finally dried under a vacuum.

**Electrophysiology Data Acquisition and Analysis.** Before the recordings, oocytes were placed into a dish containing a hyperosmotic solution (mM): 200 KCl, 20 KCl, 10 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-clamp recording. Then the oocytes were placed back into ND-96 saline (in this case without gentamicin; for composition, see previous explanation) for 10–15 minutes before ion current recording. Currents were recorded from excised, inside-out (I/O) patches. For experiments with oocytes, bath and electrode solutions contained (mM): 130 KCl, 10 CaCl₂, 5 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-clamp recording. Then the oocytes were placed back into ND-96 saline (in this case without gentamicin; for composition, see previous explanation) for 10–15 minutes before ion current recording. Currents were recorded from excised, inside-out (I/O) patches.

**Rat Cerebral Artery Myocyte Isolation.** Middle and basal cerebral arteries were isolated from adult male Sprague-Dawley rats (~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 stereomicroscope (Nikon C-PS, Tokyo, Japan) and placed into ice-cold dissociation medium (DM) with the following composition (mM): 0.16 CaCl₂, 0.49 EDTA, 10 HEPES, 5 KCl, 0.5 KH₂PO₄, 2 MgCl₂, 110 NaCl, 0.5 NaH₂PO₄, 10 NaHCO₃, 0.02 phenol red, 10 taurine, and 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 minutes in a polypropylene tube and incubated in a shaking water bath at 37°C and 60 oscillations/min for 15 minutes. Then, the supernatant was discarded, and the tissue was transferred to a polypropylene tube with 3 ml of DM containing 0.06% soybean trypsin inhibitor, 0.05% BSA, and 2% collagenase (26.6 units/ml). The tube was incubated again in a shaking water bath at 37°C and 60 oscillations/min for 15 minutes. Finally, the artery tissue pellet was transferred into a tube with 3 ml of DM containing 0.06% soybean trypsin inhibitor. Tissue-containing DM was pipetted using a series of borosilicate Pasteur pipettes having fire-polished, diminishing internal diameter tips. The procedure rendered a cell suspension containing relaxed, individual myocytes (≥5 myocytes/field using a 20× 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% soybean trypsin inhibitor and 0.06% BSA. Myocytes were used for electrophysiology up to 4 hours after isolation.

**DNA Cloning and Transcription.** The cDNA cloning and functional characterization of rat cerebral artery myocyte BK channel-forming (cbv1) subunits (AY330293) are described elsewhere (Jaggard et al., 2005). Cbv1 cDNA inserted into the pBScMXT vector was linearized with SalI and transcribed using T3 (mMessage-mMachine; Ambion, Austin, TX). The cDNA of the rat cerebral artery myocyte BK β1 subunit (FJ154955) was cloned as described in our previous work (Bukiya et al., 2009b). The BK β2/4, and BKβ1(β4TM2) chimeric cDNAs were generous gifts from Dr. Ligia Toro (University of California Los Angeles). Upon arrival, they were recloned into the pOX vector. A targeted T169A mutation was introduced into wild-type (WT) BK β1 cDNA inserted in the pOX using overlap-extension polymerase chain reaction and the Quickchange kit with pfu polymerase (Agilent Technologies, Santa Clara, CA) following the manufacturer’s instructions. Sequence of the chimeric cDNA constructs, presence of the targeted mutation, and absence of unintended mutations in the polymerase chain reaction-amplified regions of the β1–4 subunit constructs were verified by automated sequencing (Molecular Resource Center, University of Tennessee Health Science Center, Memphis, TN).

**Beta 1–4 cDNAs inserted into pOX vector were linearized, and then transcribed in vitro using either T3 or T7 polymerases (mMessage-mMachine; Ambion, Austin, TX). The cRNA was dissolved in diethyl polycarbonate-treated water at 10 ng/µl (cbv1) and 30 ng/µl (β1, β2, β3, β4, β4TM2, or β1T169A); 1 µl aliquots were stored at −70°C.

**Oocyte Extraction and cRNA Injection.** *Xenopus laevis* females were purchased from Xenopus Express (Brooksville, FL) and 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. Before the surgery, the frogs were anesthetized by placement on ice after exposure to ethyl 3-amino benzamide methanesulfonate salt (250 mg/l, pH 7.4): The oocytes were removed and kept in a Ca²⁺-free ND-96 solution (mM): 92.5 NaCl, 2 KCl, 1 MgCl₂, 5 HEPES, pH 7.5, containing 2 mg/ml collagenase (type IV; Sigma-Aldrich), at room temperature on a shaker (60 oscillations per minute) for 15 minutes to remove the follicular layer. After defolliculation, the oocytes were transferred to Ca²⁺-containing ND-96 saline (mM): 82.5 NaCl, 2 KCl, 1.8 CaCl₂, 5 HEPES, pH 7.4, supplemented with 2.5 mM sodium pyruvate and 2 mg/ml gentamicin. Injection of mRNA into the 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 to 48 hours. During this time, the injected oocytes were kept at 15°C.
a pressurized, automated DAD12 system (ALA Scientific Instruments, Farmingdale, NY) via a micropipette tip with an internal diameter of 100 μm. Experiments were performed at room temperature (20°C–22°C).

The 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) from ≥30 second of gap-free recording under each condition.

Macroscopic currents were evoked from a holding potential of 0 mV by 200 milliseconds long, 10 mV steps ranging from −150 to +150 mV. The current amplitude was averaged within 100–150 milliseconds after the start of the depolarizing step. Macroscopic conductance (G) = Gmax.V−V[1] + exp (−V + V[2]/kT). 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 (≥250 g; 10 animals) and 8- to 12-week-old male KCNMB1 KO (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) from the rat or mouse brains and cut into 1- to 2-mm-long segments. Endothelium was removed by passing an air bubble into the vessel lumen for 90 seconds before 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/min with physiologic saline solution (PSS) (mM): 3.0 KCl, 1.5 MgCl2, 1.5 CaCl2, 132 NaCl, 6.6 urea, 3.7 dextrose, and 24.6 NaHCO3. This solution was equilibrated with 6% CO2/6% O2/88% N2 to pH 7.3–7.35 at 37°C. Pial arterioles (50–100 μm in external diameter) were used to test vascular reactivity. Control PAD values were measured over a 10-minute period under basal conditions. LCA and HENA stock solutions were diluted in SSS to a final concentration of 45 μM and 1 ml of this solution was infused into the cerebral circulation via the carotid artery. A stock solution of paxillin in dimethyl sulfoxide (DMSO) was diluted in aCSF to a final concentration of 1 μM. Paxillin and 4-aminopyridine (4-AP) (0.8 mM in aCSF) were applied topically on the surface of the brain.

Chemicals

We purchased 5β-cholanic acid-3α-ol (lithocholic acid) from Steroids (Newport, RI) and methyl 3-hydroxylolan-12-en-30-oate from Hit2Lead.com (ChemBridge Corporation). All other chemicals were purchased from Sigma-Aldrich. On the day of the experiment, LCA or HENA were initially dissolved in DMSO to render stock solutions of 333 mM. 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 analysis of variance and Bonferroni’s multiple comparison test or paired Student’s t test, according to the experimental design. P < 0.05 was considered statistically significant. Data are expressed as the mean ± S.E.M., and n = number of patches/arteries/pial arterioles. Each patch was obtained from a different ocytote/myocyte, and each pressurized artery/PAD measurement was obtained from a separate animal.

Results

HENA Is a Novel, Effective Nonsteroid Activator of β1 Subunit–Containing Recombinant BK Channels. Among 18 chalone steroids tested, LCA was the most effective activator of β1-containing BK channels (Dopico et al., 2002; Bukiya et al., 2008a). Thus, in search of 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-hydroxylolan-12-en-30-oate (ID 5808244; Fig. 1A, bottom structure) as the lead compound. This compound is not a steroid, yet it contains all the structural features previously identified as necessary for chalone steroid activation of BK channels: C3-hydroxyl, hydrophobic nucleus and polar lateral chain (Dopico et al., 2002, 2012). The highly hydrophobic methyl 3-hydroxylolan-12-en-30-oate required dissolution in pure DMSO before further dissolution in saline solutions for biologic 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-hydroxylolan-12-en-30-oate and LCA, we performed a two-step organic chemical reaction to
The nonsteroid 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 in the HENA structure are shown in light gray.

render HENA (Fig. 1A, bottom structure; Supplemental Fig. 1). With a carboxylate similar to that of LCA (pKa ~5.5), the HENA molecule is expected to remain significantly ionized at physiologic pH ~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 cholan steroids (Dopico et al., 2002; Bukiya et al., 2008a). Therefore, HENA exhibits all main structural features considered necessary for BK channel activation via the BKβ1 subunit cholate steroid site (Bukiya et al., 2008a, 2011).

To determine HENA’s ability to activate BK channels, we first studied ligand action on recombinant cbv1 + β1 channels heterologously expressed in *Xenopus* oocytes. We cloned both the BK channel–forming cbv1 (AY330293) and accessory β1 (FJ154955) subunits from freshly isolated rat cerebral artery myocytes; the resulting recombinant BK complex represented an ideal model of the native BK channel (Bukiya et al., 2009b). In our experiments, we used I/O patches with the membrane potential and free Ca2+ set at values similar to those reported in the resting and contracting cerebral artery myocyte (Pérez et al., 2001). Considering that 1) HENA has a high structural similarity with LCA (Fig. 1), and 2) LCA selectively activates β1 subunit–containing BK channels (Bukiya et al., 2009a), we decided to determine whether HENA-induced BK channel activation required the presence of BK β1 subunits. We performed electrophysiologic recordings in I/O membrane patches from *Xenopus* oocytes expressing heteromic cbv1 + β2, β3, or β4 subunits, or homomic cbv1 channels under identical conditions of transmembrane voltage (Vm = −40 mV) and Ca2+ (10 μM). In all cases, the resulting ion current phenotype was confirmed by characteristic BK current kinetics (Supplemental Fig. 2) or distinctive pharmacologic features, as we previously described in detail elsewhere (Bukiya et al., 2009a). In contrast to their cbv1 + β1 counterparts, homomic cbv1 channels were consistently resistant to HENA, even when this compound was tested at 150 μM (Fig. 5, A, bottom right panel, and B), which corresponds to EC50 for HENA activation of β1-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 β1 subunits.

Moreover, heteromers made of cbv1 + β2, β3, or β4 subunits were all HENA-resistant (Fig. 5, A, bottom left and top panels, and B). Thus, as previously found for LCA (Bukiya et al., 2009a), BK β2, β3, and β4 failed to substitute for β1 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 β1 behaves as a specific sensor of HENA, likely involving the cholate steroid-recognition site recently identified in this protein (Bukiya et al., 2011).
the BK β1 protein region targeted by HENA. In the chimeric construct termed β4TM21, the BK β4 TM2 domain was substituted by the TM2 region from BK β1. The presence of functional β4TM21 coexpressed with cbv1 was confirmed by the resistance of the current to iberiotoxin (100 nM) block, which is due to the presence of the BK β4 extracellular loop region (Meera et al., 2000; Bukiya et al., 2008b).

Under experimental conditions similar to those used to probe HENA on WT β1 + cbv1, 45 μM HENA applied to the cytosolic side of I/O patches evoked a robust and reversible increase in BK NPo in all cases (Fig. 6, A and C). Remarkably, this effect was identical to that obtained with HENA and cbv1 + WT β1 channels (Fig. 2, B, D, and E), indicating that β1 TM2 is the channel region that senses the HENA presence, rendering the activation of BK channels.

The choleane-sensing site in the BK β1 TM2 domain includes Thr169, a residue that is distinct to BK β 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 β1 also required Thr169.

Thus, we coexpressed cbv1 with BK β1T169A and evaluated HENA action under experimental conditions identical to those used to evaluate the nonsteroidal agent on cbv1 + β4TM21 and cbv1 + WT β1 channels (see previous discussion). In
all cases, application of HENA, whether at 45 μM (Fig. 6, B and C) or 150 μM (unpublished data), onto I/O membrane patches expressing cbv1 + β1 subunits expressed in X. laevis I/O membrane patches. Voltage steps of 200 milliseconds duration were applied from −150 to +150 mV in 10 mV increments from $V_{\text{holding}} = 0$ mV; [Ca²⁺]free = 10 μM. (B) $G/G_{\max}$−$V$ curves from cbv1 + β1 channels at 10 μM [Ca²⁺]free show a parallel leftward shift in the presence of 45 μM HENA when compared with control. $V_{1/2}$ denotes the transmembrane voltage ($V_m$) at which $G/G_{\max} = 0.5$. Each data point represents the averaged from ≥4 patches.

**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 milliseconds duration were applied from −150 to +150 mV in 10 mV increments from $V_{\text{holding}} = 0$ mV; [Ca²⁺]free = 10 μM. (B) $G/G_{\max}$−$V$ curves from cbv1 + β1 channels at 10 μM [Ca²⁺]free show a parallel leftward shift in the presence of 45 μM HENA when compared with control. $V_{1/2}$ denotes the transmembrane voltage ($V_m$) at which $G/G_{\max} = 0.5$. Each data point represents the averaged from ≥4 patches.

HENA Activates Native Cerebral Artery Myocyte BK Channels. To determine whether HENA was able to target β1-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 Ca²⁺ were set at values within the range found in cerebrovascular myocytes during contraction: −40 to −30 mV and 3 μM free Ca²⁺ (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 μM) solution reversibly increased NPo in a concentration-dependent fashion, with $EC_{50} = 46 ± 6$ μM and $E_{\max} \sim 300$ μM (Fig. 7, B and C). These values are similar to those reported for HENA activation of recombinant cbv1 + β1 channels expressed in amphibian membranes (Fig. 2E) and LCA activation of rat cerebrovascular smooth muscle BK channels (Fig. 7) (Bukiya et al., 2007). At HENA $E_{\max}$, however, NPo reached 220% of control, which is significantly higher than that reached at LCA $E_{\max}$ (Fig. 7, B and C). Thus,
as found for recombinant channels, HENA is more efficacious than LCA in activating native cerebrovascular myocyte BK channels.

It is noteworthy that the HENA-induced increase in NPo was observed in membrane patches that were excised from the myocyte >5 minutes before applying the 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, the qualitative and quantitative similarities in HENA action on recombinant versus native BK channels suggest that the likely different proteolipid environments in amphibians versus mammalian cell membranes do not affect HENA activation of β1-containing BK channels, underscoring that potentiation of BK channel activity is due to a direct interaction between the HENA molecule and the BK β1 protein itself.

HENA Evokes Dilation of Smooth Muscle BK β1 Subunit–Containing Cerebral Arteries. To determine the impact of smooth muscle BK channel targeting by HENA on organ function, we evaluated drug action on the diameter of pressurized middle cerebral arteries from rats. The vessel dissection, de-endothelization, and pressurization are described...
in Materials and Methods. After myogenic tone development at 60 mm Hg, the arteries were sequentially probed with the control (DMSO-containing) and HENA- or LCA-containing solutions. At the end of each experiment, the artery was perfused with Ca\(^{2+}\)-free PSS to assess the passive arterial diameter.

As previously reported elsewhere (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 (\(\leq 10\) minute) application of HENA (3–45 \(\mu M\)) produced an immediate increase in arterial diameter, which was sustained as far as HENA was present in the perfusing solution; it fully recovered to pre-HENA values after washout of HENA with compound-free PSS (Fig. 8A). This HENA action was concentration dependent, with maximal artery dilation being observed in the presence of 45 \(\mu M\) HENA: 95\% of passive arterial diameter and an averaged 15\% increase in artery diameter from pre-HENA diameter values (Fig. 8, A and B). This HENA action is expected to evoke a marked increase in cerebral blood flow (~45\%) because changes in artery diameter are related to changes in cerebral blood flow by a factor of ~3 (Gourley and Heistad, 1984).

Finally, HENA-induced in vitro dilation of cerebral arteries was prevented by selective block of the BK channels with 1 \(\mu M\) paxilline (Fig. 8, C and D) (Strøbaek et al., 1996). Collectively: 1) this outcome, 2) with the arteries being de-endothelized before pressurization, and 3) with smooth muscle cells being 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, the HENA-induced increase in arterial diameter was significantly higher than that evoked by LCA (Fig. 8, A and B), paralleling the HENA versus 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 Ca\(^{2+}\) levels in cerebral artery smooth muscle, with 45 \(\mu M\) LCA being effective and HENA not (Supplemental Fig. 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 (\(KCNMB1-K/O\)) versus \(\beta 1\) subunit-containing (WT C57BL/6) mice. As found with rat cerebral arteries, 45 \(\mu M\) HENA caused a sustained yet fully reversible increase in the diameter of WT mouse cerebral arteries (~10\% from diameter before HENA application) (Fig. 9, A and C). In sharp contrast, HENA consistently failed to dilate the arteries from \(KCNMB1-K/O\) mice (\(n = 5\)) (Fig. 9, B and C), indicating that the presence of smooth muscle BK \(\beta 1\) subunits in cerebral arteries is essential for HENA-induced vasodilation.

**HENA 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 SSS (1 ml) caused a mild (<5%) and transient arteriolar dilation, which fully disappeared within 4 minutes after infusion (Fig. 10A). In contrast, 45 μM of LCA or HENA rendered up to a 10% additional dilation (i.e., on top of that evoked by vehicle). LCA- and HENA-induced dilations were 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 4th through the 10th minutes 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 ± 2.5 versus 69 ± 4.1 mm Hg before HENA intracarotid infusion and during 4–6th minutes after 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 μM on the brain surface. Paxilline application caused robust constriction of arterioles, with up to 15% reduction in diameter.
from prepaxilline values (Fig. 10B). This result underscores the key role of BK channels in limiting vessel constriction. When compared with control (DMSO-containing SSS), carotid artery infusion of 45 μM 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 prepaxilline 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-AP. At submillimolar to low millimolar concentrations, AP blocks most K<sub>V</sub> 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 the presence of HENA + paxilline, carotid infusion of HENA in the presence of 4-AP caused arteriolar dilation that was significantly higher compared with infusion of vehicle-containing SSS (Fig. 10D). Moreover, HENA-induced dilation reached 10% on top of the control value, which was identical to the HENA-induced dilation determined in the absence of 4-AP (Fig. 10, A and D).

These results indicate that K<sub>V</sub> 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 the arteriole viability after several experimental manipulations.
Discussion

It has been consistently documented that β subunits of type 1 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 β1 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. Our present data reveal for the first time that HENA, an exogenous and nonsteroidal agent, evokes a robust dilation of resistance size in cerebral arteries both in vitro and in vivo by smooth muscle BK channel activation. The strong quantitative similarities between the 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 β1 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 β1-containing BK channels.

Before our discovery of HENA, a few ligands have been claimed to require the presence of BK β1 subunits to increase BK channel activity. Among physiologic, endogenous ligands, 17β-estradiol at low micromolar levels has been reported to increase the activity of recombinant BK constructs made of hslo α (from human myometrium) + β1 subunits while failing to activate homomeric hslo α channels. This study also showed [3H]17β-estradiol-specific binding and bovine serum albumin–bound fluorescent 17β-estradiol labeling in membranes expressing BK α and β1 subunits but not in membranes expressing solely α subunits (Valverde et al., 1999). A later study, however, demonstrated that submicromolar levels of 17β-estradiol modulated BK activity in absence of BK β1 (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 results in cerebral artery dilation, a steroid action that requires selective ligand recognition by a steroid-recognition site identified in the BK β1 TM2 (Bukiya et al., 2007, 2011). Due to their steroidal character, LCA and related cholanes exert numerous biologic 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 taurolithocholic acid 3-sulfate in pancreatic acinar cells (Gerasimenko et al., 2006), we showed here that μM LCA raised overall cytosolic Ca\(^{2+}\) in endothelium-free cerebral artery, an effect not observed with HENA (Supplemental Fig. 9).
Thus, any possible use of LCA as a vasodilator might require additional interventions to reduce a bile-acid-induced increase in \( Ca^{2+}_i \), as this increase would oppose smooth muscle relaxation and vasodilation. More generally, the widespread side effects secondary to choline 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 physiologic 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 dehydrosyasaponin-I (DHS-I), derived from the medicinal herb *Desmodium adscendens*, has been shown to activate \( \beta_1 \) subunit–containing BK channels at nanomolar 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), which drastically limits the delivery of DHS-I to myocyte BK channels via circulation. This limitation does not apply to HENA, as demonstrated by our 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 can activate \( \beta_1 \)-containing and neuronally abundant, \( \beta_4 \)-containing BK channels (Morimoto et al., 2007). The benzimidazolone NS1619 [4-(trifluoromethyl)-2-[5-(trifluoromethyl)-2,3-dihydrobenzimidazol-1-yl]phenol] (Papassotiriou et al., 2000), the benzofuroindole CTBIC...
[4-chloro-7-(trifluoromethyl)-10H-benzo[furo[3,2-b]indole-1-carboxylic acid], and the tetrahydroxquinoline termed compound Z \([\text{Z}\{3\text{aR,4S,9bS}\}-4\text{-}(\text{Naphthalen-1-yl})-3\text{a,4,5,9b-tetrahydro-3H-cyclopenta[\text{c}]quinoline-8-carboxylic acid}\} \text{(Lee et al., 2012; Ponte et al., 2012)}\) 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 KC- precontracted isolated thoracic aortic rings via BK \(\beta_1\) subunits (Caldorena et al., 2008). Likewise, it remains unknown whether NS11021 \([1\text{-}\{3,5\text{-bis-trifluoromethyl-phenyl}\}3\text{-}(4\text{-bromo-2\text{-}(1\text{-}\text{H-tetrazol-5-yl})\text{-phenyl})\text{-thiourea}\} \text{and related anthraquinones activate BK channels} \text{(Roy et al., 2012)}\) via BK \(\beta_1\) and, if so, whether other BK \(\beta\)s can substitute for BK \(\beta_1\) 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 BK1-containing BK channels. From a basic research standpoint, HENA can be used as a pharmacologic tool alternative to LCA for detecting BK1-containing BK channels and targeting the BK1 subunit steroid-sensing site in biologic preparations. Consideration of HENA and related compounds in clinical settings, however, requires some additional discussion. HENA belongs to a large family of naturally occurring penta cyclic triterpenes (PT) derived from herbal sources. Several derivatives having the PT skeleton are antitumor agents and inhibitors of glycolen phosphorylase and Na*/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 \(\text{Kv}\) TM6 channels other than BK (Fig. 1D). 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 as 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 \(\mu\)M HENA whether applied in vitro to a de-endothelialized artery or in vivo through intracarotid infusion increases cerebral artery diameter by ~15\% \text{(Fig. 10).} This change is expected to evoke a robust increase in cerebral blood flow \((\text{by ~}45\%\text{)}\) (Gourley and Heistad, 1984), and it would change is expected to evoke a robust increase in cerebral blood flow \((\text{by ~}45\%\text{)}\) (Gourley and Heistad, 1984), and it would.
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+β(1–4) subunit expression in *Xenopus laevis* oocytes. A. Representative current records from I/O macropatches expressing different BK channel subunit combinations (cbv1±β1, β2, or β4). 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+β3 heteromers results in longer channel openings when compared to those from homomeric cbv1. Vm=+40mV, free [Ca$^{2+}$]=10 μM.
Suppl. Fig. 3. Ratiometric detection of changes in arterial wall [Ca2+]i in presence of LCA vs. HENA.
A. Original trace showing LCA-induced increase in [Ca2+]. B. Original trace showing lack of [Ca2+]i increase by HENA, with high KCl being used as positive control. C. Averaged changes in arterial wall [Ca2+]i evoked by LCA, HENA, and KCl. *Different from LCA-induced increase in arterial wall [Ca2+]i (P<0.05). In A-C, data were obtained in the presence of BK channel selective blockade by 1 μM paxilline.
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 µM 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 µM 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 hyperswitch (Ionoptix, Milton, MA) for ratiometric detection of free [Ca$^{2+}$] levels within each arterial segment.