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

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ABSTRACT

The Ca\(^{2+}\)/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 (EC\(_{50}\) 1) similar to and an efficacy (\(\times 2.5\) potentiation) significantly greater than that of LCA.

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 Ca\(^{2+}\)/gated K\(^+\) (BK) channels result from the association of four identical α (slo1) subunits, which are ubiquitously distributed (Ghatta et al., 2006; Salkoff et al., 2006). In most tissues, however, a homotetramer is accompanied by small, two transmembrane (TM2)-spanning accessory (β1–4) subunits that are encoded by four separate genes: KCNMB1–4 (Orio et al., 2002). Remarkably, KCNMB1 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 Ca\(^{2+}\) entry, limiting constriction...
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 KO (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 KO 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 vasocostriction underlies numerous pathologic conditions, including cerebral vasospasm, ischemia after aneurysmal subarachnoid hemorrhage (Jordan and Nyquist, 2010), the reversible cerebral vasocostriction syndrome associated with nonaneurysmal subarachnoid hemorrhage, pregnancy, or exposure to certain drugs (Sattar et al., 2010), posttraumatic cerebral vasospasm after traumatic brain injury (Shahlaie et al., 2009), and abrupt-onset severe headaches (Ju and Schwedt, 2010). In addition, cerebral vasocostriction 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 chalone 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 KO 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-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 Molecular Operating Environment (MOE) 2006.08 (Chemical Computing Group, Montreal, QC, Canada).

**Chemistry**

We hydrolyzed 3’-hydroxyolean-12-en-29-oic acid methyl ester (compound 1) to obtain 3’-hydroxyolean-12-en-29-oic acid (compound 2) (Supplemental Fig. 1). Purified acid (2) was further converted to the corresponding sodium salt of 3’-hydroxyolean-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 \([\text{H}]\) and \([\text{C}^1\text{C}]\) 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 Compound 2 (2S,4aS,6aS,6bR,8aR,10S,12aR,12bR,14bR)-10-Hydroxy-2,4a,6a,8b,12a-Heptamethyl-1,2,3,4,5,6,6a,6b,7,8,9,10,11,12a,12b,13,14b-Icosahydropicene-2-Carboxylic Acid).** Compound 1 (0.085 mM, 40 mg) was dissolved in 10 mL of ethanol. After 300 mg of KOH dissolved in 2 mL of water had been added, the mix was refluxed for nearly 8 hours. The reaction mixture was then cooled and carefully acidified with diluted HCl. The Pharmacologic Vasodilation via the BK β1 Steroid Site
mixture was extracted with chloroform. The chloroform layer was dried over anhydrous Na2SO4, then the chloroform was removed under reduced pressure. The crude product was purified by flash chromatography (ethyl acetate/hexanes; 1:9).

Yield 34%; 1H NMR (CDCl3). Compound 2 was obtained in 34% yield, its structure being confirmed by 1H NMR (CDCl3): δ 5.32 (t, J = 10.0 Hz, 1H), 4.72–4.75 (m, 1H), 1.46–1.48 (m, 1H, 2CH3 groups were merged in the multiplet), 0.90–0.72 (m, 8H, 2CH3 groups were merged in the multiplet); 13C 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, 14.93.

Preparation of Compound 3. 3-hydroxyolean-12-en-30-oate, was referred to as “HENA” and was used to test biologic activities.

cDNA Cloning and Transcription

The cDNA cloning and functional characterization of rat cerebral artery myocyte BK channel-forming (cblv1) subunits (AY330293) are described elsewhere (Jaggar et al., 2005). Cblv1 cDNA inserted into the pBSmXT vector was linearized with Sall 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 cDNAs inserted into pOX vector were linearized and then transcribed using T3 (mMessage-mMachine; Ambion, Austin, TX). The cDNA cloning and functional characterization of rat cerebral artery myocyte BK channel-forming (cblv1) subunits (AY330293) are described elsewhere (Jaggar et al., 2005). Cblv1 cDNA inserted into the pBSmXT vector was linearized with Sall and transcribed using T3 (mMessage-mMachine; Ambion, Austin, TX). The cDNA cloning and functional characterization of rat cerebral artery myocyte BK channel-forming (cblv1) subunits (AY330293) are described elsewhere (Jaggar et al., 2005). Cblv1 cDNA inserted into the pBSmXT vector was linearized with Sall and transcribed using T3 (mMessage-mMachine; Ambion, Austin, TX).

Sequence of the chimeric cDNA constructs, presence of the targeted T169A mutation was introduced into wild-type (WT) BK β1 subunit (FJ154955) cDNAs inserted into pOX vector were linearized and then 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 cDNAs inserted into pOX vector were linearized and then transcribed using T3 (mMessage-mMachine; Ambion, Austin, TX). The cDNA cloning and functional characterization of rat cerebral artery myocyte BK channel-forming (cblv1) subunits (AY330293) are described elsewhere (Jaggar et al., 2005). Cblv1 cDNA inserted into the pBSmXT vector was linearized with Sall and transcribed using T3 (mMessage-mMachine; Ambion, Austin, TX).

Electrophysiology Data Acquisition and Analysis

Before the recordings, oocytes were placed into a dish containing a hypertonic solution (mM): 200 K+ aspartate, 20 KCl, 1 MgCl2, 10 EGTA, 10 HEPES, pH 7.4, for 10 minutes. With this treatment, the oocytes shrink, allowing the removal of the vitelline layer with forces 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 ionic current recording. Currents were recorded from excised, inside-out (I/O) patches. For experiments with oocytes, bath-bath and electrode solutions contained (mM): 130 K+ gluconate, 5 EGTA, 22.5 MgCl2, 15 HEPES, and 1.6 N-(2-Hydroxymethyl) ethylenediaminetetraacetic acid (HEDTA), pH 7.35. For experiments with myocytes, KCl substituted for K+ gluconate.

In all experiments, the free Ca2+ in solution was adjusted to the desired value by adding CaCl2. When the desired free Ca2+ did not exceed 1 μM, HEDTA was omitted from the solution, and the final MgCl2 was set to 1 mM. In all cases, the nominal free Ca2+ was calculated with MaxChelator Sliders (C. Patton, Stanford University, Stanford, CA) and was validated experimentally using Ca2+–selective electrodes (Corning Incorporated Science Products Division, Corning, NY) (Dopico, 2003).

Patch-clamp electrodes were pulled from glass capillaries (Drummond Scientific Co.). Immediately before recording, the tip of the electrode was fire-polished on a microforge (World Precision Instruments, Sarasota, FL) to give resistances of 8–10 MΩ when filled with extracellular solution (for composition, see previous explanation). An agar bridge with gluconate or chloride as the main anion was used as the 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 the 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 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 Gmax/V plots were fitted to a Boltzmann function of the type G(V) = Gmax/V1 + exp(−V − V1/2)/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 (≤250 g; 10 animals) and 8- to 12-week-old male KCMN61 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): 119 NaCl, 4.7 KCl, 1.2 KH2PO4, 1.6 CaCl2, 1.2 MgSO4, 0.023 EDTA, 11 glucose, and 24 NaHCO3. This solution was equilibrated with 6% CO2/6% O2/88% N2 to pH 7.35–7.35 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 paxilline in dimethyl sulfoxide (DMSO) was diluted in aCSF to a final concentration of 1 μM. Paxilline and 4-aminopyridine (4-AP) (0.8 mM in aCSF) were applied topically on the surface of the brain.

**Pharmacologic Vasodilation via the BK β1 Steroid Site**

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, bottom structure) as the lead compound. The screening yielded methyl 3-hydroxyolean-12-en-30-oate required dissolution in pure DMSO before stock solutions of 333 μM. Stocks were sonicated for 30 minutes and then further diluted with either either physiologic 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 oocyte/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-hydroxyolean-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-hydroxy, 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 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-hydroxyolean-12-en-30-oate and LCA, we performed a two-step organic chemical reaction to
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 \( \approx 7.3–7.4 \). Thus, flexible alignment of ionized LCA and HENA molecules showed high degree of overlap at both their C3-hydroxyl and carbonyl 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 \( \text{cbv1} + \beta 1 \) channels heterologously expressed in \( \text{Xenopus oocytes} \). We cloned both the BK channel–forming \( \text{cbv1} \) (AY330293) and accessory \( \beta 1 \) (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 Ca\(^{2+}\) set at values similar to those reported in the resting and contracting cerebral artery myocyte (Jaggar et al., 1998; Knot and Nelson, 1998; Pérez et al., 2001). After excision, each patch was exposed to the control (solvent-containing) solution, and BK activity was continuously recorded for no less than 30 seconds. Application of the LCA-containing (1–1,000 \( \mu \)M) solution reversibly increased NPo in a concentration-dependent fashion: \( EC_{50} = 46.9 \pm 4.1 \mu \text{M}, E_{\text{max}} \approx 300 \mu \text{M} \) (Fig. 2, A, D, and E). These values are similar to those reported with LCA on this system and are 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: \( EC_{50} = 53.4 \pm 3.9 \mu \text{M} \) and \( E_{\text{max}} \approx 300 \mu \text{M} \) (Fig. 2, B, D, and E). For both LCA and HENA, drug potentiation of the BK channel activity did not depend on basal, predrug 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 \( E_{\text{max}} \) reaching 2.5 times that of control.

It is noteworthy that HENA-induced BK channel activation remained fairly constant within a wide voltage range (\( \approx -150 \) to \( \approx +150 \text{mV} \)), as documented by a parallel leftward shift in \( E_{\text{Gmax}} \) plots from macroscopic \( \text{cbv1} + \beta 1 \) currents in the presence versus absence of HENA (Fig. 3, A and B). On the other hand, HENA-induced BK channel activation was identical at submicromolar (0.3 \( \mu \)M) and micromolar (10 \( \mu \)M) levels of free Ca\(^{2+}\) (Fig. 4, A and B). Collectively, our results demonstrate that HENA-induced BK channel activation is voltage independent and observed at levels of Ca\(^{2+}\) found 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 \( \beta 1 \) subunit–containing BK channels (Bukiya et al., 2009a), we decided to determine whether HENA-induced BK channel activation required the presence of BK \( \beta 1 \) subunits. We performed electrophysiologic recordings in I/O membrane patches from \( \text{Xenopus oocytes} \) expressing heteromic \( \text{cbv1} + \beta 2, \beta 3, \) or \( \beta 4 \) subunits, or homomeric \( \text{cbv1} \) channels under identical conditions of transmembrane voltage (\( V_m = -40 \text{ mV} \)) and Ca\(^{2+}\) (10 \( \mu \)M). 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 \( \text{cbv1} + \beta 1 \) counterparts, homomeric \( \text{cbv1} \) channels were consistently resistant to HENA, even when this compound was tested at 150 \( \mu \)M (Fig. 5, A, bottom right panel, and B), which corresponds to \( EC_{90} \) for HENA activation of \( \beta 1 \)-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 \( \beta 1 \) subunits.

Moreover, heteromers made of \( \text{cbv1} + \beta 2, \beta 3, \) or \( \beta 4 \) 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 \( \beta 2, \beta 3, \) and \( \beta 4 \) failed 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).

**HENA 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 the TM2 of \( \beta 1 \), we used chimeric \( \beta 1/\beta 4 \) subunits to identify...
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 cholane-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 \( \mu M \) (Fig. 6, B and C) or 150 \( \mu M \) (unpublished data), onto I/O membrane patches expressing cbv1 + \( \beta 1 \) failed to modify BK NPo (\( n = 4 \) for each HENA concentration). These negative data indicate that Thr169, a residue unique to BK \( \beta \) subunits of type 1 and critical for providing cholesterol 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 cholesterol steroid site (see Discussion).

**HENA 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\(^{2+}\) were set at values within the range found in cerebrovascular myocytes during contraction: \(-40 \) to \(-30 \) mV and 3 \( \mu M \) free Ca\(^{2+}\) (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 \( E_{\text{max}} \sim 300 \) \( \mu M \) (Fig. 7, B and C). These values are similar to those reported for HENA activation of recombinant cbv1 + \( \beta 1 \) 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 \( E_{\text{max}} \), however, NPo reached 220% of control, which is significantly higher than that reached at LCA \( E_{\text{max}} \) (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

![Fig. 5. BK β1s fail to substitute for β1 in providing HENA sensitivity to the BK complex. (A) Unitary current records obtained in I/O patches from X. laevis oocytes that express BK channels of different subunit composition. V_m = −40 mV, [Ca^{2+}]_{free} = 10 μM. (B) Average increase in BK activity (NPo) in the presence of 150 μM HENA. Each point was obtained from no less than 4–5 patches. *Different from cbv1 (P < 0.05).](image-url)
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 (<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 \(\beta1\) subunit targeting by this ligand, we evaluated HENA action on the arterial diameter of de-endothelized, pressurized cerebral arteries from \(\beta1\) subunit-lacking (\(\text{KCNMB1 K/O}\)) versus \(\beta1\) 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 \(\text{KCNMB1 K/O}\) mice (\(n = 5\)) (Fig. 9, B and C), indicating that the presence of smooth muscle BK \(\beta1\) 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.

Fig. 7. HENA increases the activity of rat cerebral artery myocyte BK channels more effectively than LCA. (A and 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 μM LCA (A) or 45 μM HENA application (B). $V_{m} = −40$ mV, $[Ca^{2+}]_{free} = 3$ μM. (C) concentration response curves for HENA and LCA. Each point represents the average of no less than three patches, each patch excised from a different oocyte. *Different from LCA ($P < 0.05$). (D) Concentration-response curves where the drug-induced BK channel activation at a given concentration is normalized to the drug’s maximal effect show that HENA and LCA have a similar EC$_{50}$. 

$$\text{EC}_{50} = 41.1 \mu M \quad \text{LCA}$$

$$\text{EC}_{50} = 46.0 \mu M \quad \text{HENA}$$
from paxilline 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^+ (K_V) 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_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 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_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 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 Ca2+ i in endothelium-free cerebral artery, an effect not observed with HENA (Supplemental

Fig. 9. HENA fails to dilate pressurized arteries from BK β1 subunit-lacking (KCNMB1 K/O) mice. Cerebral artery diameter traces show that acute application of 45 μM HENA causes sustained diameter increase (dilation) in arteries from BK β1 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 with pre-HENA levels (hollow bars). *Different from diameter before HENA application (P < 0.05).
Thus, any possible use of LCA as a vasodilator might require additional interventions to reduce a bile-acid-induced increase in Ca\(^{2+}\), as this increase would oppose smooth muscle relaxation and vasodilation. More generally, the widespread side effects secondary to cholesteroid-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 dehydrosoyasaponin-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-benzofuro[3,2-b]indole-1-carboxylic acid], and the tetrahydroquinoline termed compound Z [3aR,4S,9bS]-4-(Naphthalen-1-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline-8-carboxylic acid] (Lee et al., 2012; Ponte et al., 2012) all induce BK channel activation in absence of BK β1, with drug action being likely mediated by the ubiquitously distributed BK α subunit. It remains unknown whether NS1619 amido derivatives promote relaxation of KCrai-precontracted isolated thoracic aortic rings via BK β1 subunits (Calderon et al., 2008). Likewise, it remains unknown whether NS11021 [1-(3,5-bis-trifluoromethyl-phenyl)-3-(4-bromo-2-(1H-tetrazol-5-yl)-phenyl)-thiourea] and related antraquinonates activate BK channels (Roy et al., 2012) via BK β1 and, if so, whether other BK βs can substitute for β1 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 β1-containing BK channels. From a basic research standpoint, HENA can be used as a pharmacologic tool alternative to LCA for detecting β1-containing BK channels and targeting the BK β1 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
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