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Ca²⁺- and voltage-gated potassium (BK) channel activators in the 5β-cholanic acid-3α-ol analogue series with modifications in lateral chain

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Abstract

Large conductance, calcium- and voltage-gated potassium (BK) channels regulate various physiological processes and represent an attractive target for drug discovery. Numerous BK channel activators are available. However, these agents usually interact with the ubiquitously distributed channel-forming subunit and thus cannot selectively target a particular tissue. Here, we performed structure-activity relationship study of lithocholic acid (LCA), a cholane that activates BK channels via the accessory BK β1 subunit. The latter protein highly abundant in smooth muscle but scarce in most tissues. Modifications in the LCA lateral chain length and functional group yielded two novel smooth muscle BK channel activators, both having a small volume and a net negative charge in the substituent radical at C24. Our data provide detailed structural information that will be used to advance a pharmacophore in search of β1 subunit-selective BK channel activators. These compounds are expected to evoke smooth muscle relaxation, which would be beneficial in the pharmacotherapy of prevalent human disorders associated with increased smooth muscle degree of contraction, such as systemic hypertension, cerebral or coronary vasospasm, bronchial asthma, bladder hyperactivity, and erectile dysfunction.

Keywords

MaxiK channel; structure-activity relationships; analogue series; BK β1 subunit; steroids

Introduction

Large conductance, Ca²⁺- and voltage-gated K⁺ (BK) channels link intracellular Ca²⁺ homeostasis to cell plasma membrane excitability.^[1] Thus, BK channels are critical for numerous physiological processes, including neuronal firing, neurotransmitter release, hormonal secretion, tuning of cochlear hair cells, immunity,^[2–3] regulation of apoptosis^[4]

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and cancer cell dissemination.^[5] In smooth muscle cells, outward K⁺ current generated by BK channel activity negatively feed-backs on Ca²⁺ entry into the cell, opposing smooth muscle cell contraction and promoting myocyte relaxation.^[6] Thus, pharmacological activation of smooth muscle BK channels constitute an effective tool to evoke smooth muscle relaxation, which could open a new avenue in the pharmacotherapy of a wide variety of prevalent human conditions associated with smooth muscle increased degree of contraction, including bronchial asthma, systemic hypertension, cerebral and coronary vasospasm, and overactive urinary bladder.^[7–9]

Smooth muscle BK channels are heterooligomers resulting from the association of four channel-forming α (or slo1) subunits (encoded by *Slo1* or *KCNMA1*) with small, accessory β 1 proteins (encoded by the *KCNMB1* gene). In absence of accessory beta subunits, slo1-targeting seems to be involved in BK channel activation by a variety of naturally occurring and synthetic compounds,^[8, 10–11] including the pioneer benzimidazolones NS004 and NS1619^[12–13] and their derivative Cym04,^[14] the thiourea 1-(3,5-bis-trifluoromethyl-phenyl)-3-[4-bromo-2-(1H-tetrazol-5-yl)-phenyl]-thiourea (NS11021),^[15] pimelic acid and related terpenes,^[16] the flavonoid puerarin,^[17] the tetrahydroquinoline (3*a*R,4*S*,9*b**S*)-4-(Naphthalen-1-yl)-3*a*,4,5,9*b*-tetrahydro-3*H*-cyclopenta[*c*]quinoline-8-carboxylic acid,^[9] and the benzofuroindole 4-chloro-7-(trifluoromethyl)-10*H*-benzofuro[3,2-*b*]indole-1-carboxylic acid.^[18] The ubiquitous location of slo1 BK channel-forming proteins and the involvement of BK channels in a wide variety of physiological and pathophysiological processes^[6, 19] raise the concern that effective BK channel activators that act via recognition by slo1 subunits, while likely effective in regulating smooth muscle contraction, may also render a wide-variety of unwanted side effects due to BK channel activation outside the smooth muscle.

In contrast to the widespread and abundant distribution of BK α subunits, β 1 proteins are distinctively abundant in smooth muscle.^[6, 20] Therefore, selective pharmacological activation of β 1 subunit-containing BK channels is postulated to evoke smooth muscle relaxation while causing a very small number of BK channel-mediated side effects outside the smooth muscle, if any. Unfortunately, a very limited set of compounds have been reported to require the presence of BK β 1 subunits to evoke channel activation. The first ever reported is a set of triterpenoid glycosides isolated from the medicinal herb *Desmodium adscendens*.^[21] The most potent of these compounds is dehydrosoyasaponin I (DHS-I), which reversibly increased the open probability of BK channels from bovine tracheal smooth muscle at concentrations as low as 10 nM. When tested at E_{max} (100–1,000 nM), this compound caused an ~125-fold increase in the open probability of BK channels reconstituted into artificial lipid bilayers.^[22] However, BK channel activation by DHS-I requires compound application from the intracellular side of the membrane,^[21] which represents a shortcoming for the administration of DHS-I in the intact organism. In addition, it remains currently unknown whether DHS-I action is selective on β 1-containing BK channels or, rather, BK β subunits other than β 1 may embolden the slo1 channel complex with DHS-I sensitivity, which would result in widespread targeting of BK channels in tissues other than smooth muscle. The aforementioned limitations would drastically reduce the use of DHS-I and derivatives as smooth muscle relaxants in the clinical setting.

Cholane steroids represent another class of naturally occurring compounds that target $\beta 1$ subunit-containing BK channels. Among cholane steroids, 5 β -cholan-3 α -ol or lithocholic acid (LCA) is the most effective BK channel activator, with channel activation evoked by LCA at the low micromolar range.^[23–24] At these concentrations, LCA exerts a wide variety of physiological effects other than reducing smooth muscle contractility, including hepatotoxicity, induction of pancreatitis, and potential enteric carcinogenesis, the targets mediating these effects being not fully characterized. However, it has been reported that LCA and other bile acids interact with a variety of proteins other than BK channels: the G-protein-coupled receptor BG-37,^[25] vitamin D receptor,^[26] ryanodine and IP₃ receptors,^[27] cytosolic steroid-binding proteins, membrane transporters, and transcription factors.^[28] These bile acid-protein interactions empower LCA and related cholanes with systemic endocrine functions that include bile acid regulation of their own enterohepatic circulation, triglyceride, cholesterol, energy, and glucose homeostasis.^[29] The wide variety of receptors and organs targeted by bile acids may preclude the use of LCA in smooth muscle-relaxing pharmacotherapy. Detailed structure-activity relationship studies of cholane-based steroids, however, represent the main lead to the design of BK channel activators that target the BK $\beta 1$ subunit and may have a pharmacological profile narrower than that of LCA.

The LCA molecule possesses three distinct chemical features (Figure 1a): 1) C3 hydroxyl group in α configuration; 2) *cis* joint between A and B steroid rings; 3) C24 carboxyl group in the lateral chain. In the steroid nucleus, both, C3 α -hydroxyl group and *cis* A/B ring joint were shown to be critical for cholane activation of $\beta 1$ -containing BK channels. In particular, inversion of the C3 hydroxyl into the β -configuration or replacement of the *cis* A/B joint with the corresponding *trans* isomer resulted in poor BK channel activation.^[24] Moreover, our recent mutagenesis study pinpointed T169 and L172, L173 within the BK $\beta 1$ subunit TM2 domain as sensors of C3 α -hydroxyl and A/B *cis* joint of LCA, respectively (Figure 1b).^[30]

In contrast to the detailed aforementioned information on the structural requirements in the LCA nucleus for BK channel activation, the chemical determinants in the steroid lateral chain that participate in BK channel activation by LCA and analogs remain to be identified. In the LCA lateral chain, esterification of C24 carboxyl resulted in significant reduction in LCA action.^[24] However, nonconserved substitutions of BK $\beta 1$ subunit amino acids in the vicinity of the cholane C24 carboxyl did not necessarily change LCA-induced BK channel activation,^[30–31] suggesting that the chemical requirements in the steroid lateral chain for cholanes to activate BK channels are rather lax. This earlier finding raises the hypothesis that chemical substitutions introduced in the cholane steroid lateral chain render small ligands with sustained ability to activate BK channels. In the current study, we designed, synthesized, and tested the activity of LCA steroidal analogues to pinpoint the structural requirements in the cholane lateral change required to activate BK channels and thus, unveil effective and novel $\beta 1$ -containing BK channel activators. Compounds were evaluated by their ability to increase the steady-state activity (NPo) of smooth muscle BK channels in conventional patch-clamp electrophysiology assays after co-expression of BK α and $\beta 1$ subunits in *Xenopus laevis* oocytes, as previously described.^[7, 24, 31]

Results and Discussion

Initial modification of the LCA molecule (C24) began with the shortening and elongation of LCA isooctyl chain attached to C17 of the steroid nucleus, rendering C24 and C25 steroids, respectively (Figure 2a). Compounds **3** and **15** were generated and tested at 150 μ M, the latter being the EC (effective concentration causing 90% of maximum effect) for LCA activation of β 1-containing BK channels.^[7] BK channel activation by 150 μ M LCA (Figure 2b, c) served as positive control. We found that shortening the LCA lateral chain (compound **3**) practically abolished cholane-induced BK channel activation (Figure 2c). On the other hand, elongation of LCA lateral chain (compound **15**) resulted in BK channel activation, which was significantly less than the activation caused by 150 μ M LCA (Figure 2c). However, compound **15** evoked up to 1.8-fold increase in channel NPo (Figure 2c), with EC₅₀ undistinguishable from LCA EC₅₀ (Figure 2d). These data indicate that the length of the steroid lateral chain determines the efficacy of β 1-containing BK channel activation. Based on our channel-LCA docking model (Figure 1b), we interpret that compound **3** lateral chain being shorter than that of LCA does not enable this C23 steroid to reach critical interacting protein residues. In turn, compound **15** lateral chain, which is longer than that of LCA, can still reach interacting protein residues given the significant mobility expected from this lateral chain. The decreased efficacy of compound **15** compared to LCA could result from steric hindrance associated to accommodation of a long chain by a confined binding surface.

We next introduced a series of functional substitutions at C25 to determine whether such substitutions could override the deleterious effect of a longer side chain, with the resulting compounds having efficacy higher or, at least, similar to that of LCA. Modifications at C25 resulting in introduction of partial positive charge and/or additional volume with either positive or negative charges (compounds **13**, **16**, **18**, **21**, Table 1), however, totally ablated cholane-induced BK channel activation (Figure 3). In contrast, substitution of the carboxyl at C25 with relatively small functional groups carrying overall negative charge (compounds **14** and **19**, Table 1) sustained BK channel activation, which in both cases was not significantly different from the activation evoked by compound **15**. Thus, provided the side chain length is similar, the presence of functional groups with a small negative charge/density seems to favor BK channel activation. The differential actions of compounds **14** and **19** versus compound **18** also indicate that negative charge/density is not sufficient to activate the channel, with bulkier head groups possibly facing steric constraints at the protein binding surface (see Table 1 and further discussion below). However, all the introduced modifications at C25 resulted in lower BK channel activation when compared to that of LCA (Figure 3). These data are consistent with those shown in Figure 2 and underscore that the distance between the negatively charged atom and the steroidal ring “core” is a critical determinant for effective BK channel activation by LCA and analogues.

Considering that C23 (Figure 2) and C25 molecules (Figure 3) failed to mimic the efficacy of LCA to activate BK channels, we next generated a set of LCA analogues (compounds **7**, **11**, **12**, **17**, **22**, **23**) with modified C24 carboxyl (Figure 4a). Data show that among the C24 substitutions, only nitro- and cyano- modifications (compounds **7** and **11**) rendered BK channel activations that were similar to that evoked by LCA (Figure 4b). This outcome

parallels that from the C25 series of compounds (Figure 3). Moreover, concentration-response curves for compounds **7** and **11** were also indistinguishable from that of LCA (Figure 4c). From the results shown in Figure 4, and group overall volume and net charge data shown in Table 1, we conclude that LCA analogs with C24 substitution are effective BK channel activators provided their functional groups at C24 combine a rather small volume (65–92 Å³) and net negative charge (−0.1 to −0.9). These ranges correspond to the compounds that satisfy both criteria: LCA, compound **7**, and **11** (Table 1). Compound **23**, while having an overall volume (0.92 Å³) falling within the range characteristic of active compounds but without negative net charge, failed to activate the channel. Conversely, compound **12** has a net negative charge but its overall group head volume (157 Å³) is much larger than those of active compounds. Thus, compound **12** was also ineffective. Other C24 substitutions (compounds **17** and **22**) did not satisfy either criteria and, thus, were ineffective. Overall, data and interpretation on the importance of negative charge and group head volume from the C24 substitution series (Figure 4) are similar to those from the C25 series (Figure 3). Inappropriate distance between the negatively charged atom(s) and the steroidal ring core in the latter group, however, determines that some compounds with a relatively small overall volume and net negative charge of the functional group in lateral chain (compound **14**, **19**) are not as effective as LCA, compound **7**, and **11** to evoke BK channel activation.

In synthesis, current structure-activity relationship study on LCA analogue series revealed chemical requirements in cholane lateral chain for effective BK channel activation. These structural requirements include 1) optimal length of the lateral chain (with C24 analogues being most effective), 2) small overall volume and 3) presence of net negative charge in the functional group at the end of the steroid lateral chain.

Our findings open new ground for developing novel BK channel activators that selectively target $\beta 1$ subunit-containing BK channels. The major step in this endeavor will represent the development of novel LCA analogs which, while keeping selectivity on $\beta 1$ subunit-containing channels complexes over homomeric slo1 or heteromeric channels resulting from association of slo1 with $\beta 2$, $\beta 3$ or $\beta 4$ subunits, show equal or better potency and efficacy than LCA, and lack structural features responsible for widespread physiological effects of LCA and related steroids. The latter task requires extensive search and testing, as in many cases the structural features of the cholane molecule required to evoke a given biological effect are currently unknown. Remarkably, modification in the iso-octyl lateral chain of LCA greatly diminished the ability of this steroid to inhibit the xenobiotic-metabolizing enzymes glutathione-S-transferase and glucuronosyltransferase in colonic epithelial cells, the activity of these enzymes being proposed to underlie some cancerogenic properties of LCA.^[32] Introduction of variant chemical groups into the steroidal nucleus and lateral chain of LCA also affected the ability of LCA analogs to bind the N-terminal 8-kDa domain of DNA polymerase β .^[33] It is tempting to speculate that modifications in LCA lateral chain could help discriminate among LCA targets, which would hopefully translate into BK channel activator(s) with fewer side effects than LCA. Should these compounds still evoke unwanted biological actions similar to those induced by LCA, replacement of the LCA

steroidal ring would be the next step in a rationale search for $\beta 1$ subunit-containing BK channel activators.

The steroidal ring core has been implicated in mediating several interactions between cholanes and targets other than BK channels. For instance, modifications in the steroidal core greatly affected the competitive binding of cholanes to the vitamin D receptor,^[26] bile acid hemolytic activity^[34] and cholestatic potency,^[35] bile acid regulation of cholesterol 7 α -hydrolase and sterol 27-hydrolase activities,^[36] bile salt recognition by human bile acid-binding protein,^[37] and the cytotoxic and bacteriostatic properties of bile.^[38] It is thus hypothesized that elimination of the steroid structure in newly developed BK channel activators diminishes off-target physiological effects.

Based on earlier developed LCA-BK $\beta 1$ subunit docking model,^[24] we designed and synthesized a set of hydroxy-alkynoic acids and their corresponding methyl esters.^[39] Although these compounds showed modest efficacy to activate BK channels, they represent the first proof-of-principle that LCA can be used as a starting point in developing non-steroidal BK channel activators. More recently, using LCA as a template, we performed similarity search of chemical structure databases and found the very promising non-steroidal lead methyl 3-hydroxyolean-12-en-30-oate, which met basic structural LCA requirements for BK channel activation. Preliminary data showed that a hydrolyzed form of this compound, sodium 3-hydroxyolean-12-en-30-oate, was more effective than LCA in activating recombinant (cbv1+ $\beta 1$) or native BK channels from rat cerebral artery myocytes. Moreover, 3-hydroxyolean-12-en-30-oate produced remarkable cerebral artery smooth muscle *in vitro* relaxation and cerebral pial arteriole *in vivo* dilation. Finally, the observed effects were attributed to methyl 3-hydroxyolean-12-en-30-oate interaction with the LCA-sensing site of the BK $\beta 1$ subunit. These preliminary findings underscore the feasibility of developing a non-steroidal, BK channel activator and smooth muscle relaxing agent based on the LCA- $\beta 1$ subunit interaction (A. N. Bukiya, J. McMillan, A. L. Fedinec, C. W. Leffler, A. L. Parrill, A. M. Dopico, *Biophysical Society 56th Annual Meeting*, 2012, San Diego, CA, February 25–29).

Conclusions

In the current SAR study of LCA analogue series we revealed the chemical requirements in the cholane lateral chain for effective activation of $\beta 1$ subunit-containing BK channels. These requirements include: 1) presence of net negative charge in the functional group at the end of the steroid lateral chain; 2) small overall volume of the negatively charged group, and 3) an optimal distance between the negatively charged atom(s) and the steroidal core (with C24 analogues being the most effective). In conjunction with a newly refined model of cholane-BK $\beta 1$ subunit interaction,^[30] present data provide detailed structural information that will be used to advance a pharmacophore in search of BK $\beta 1$ subunit-selective BK channel activators and effective smooth muscle relaxants with few side effects. These smooth muscle relaxants will have great pharmacotherapeutic potential in prevalent human disorders linked to smooth muscle hypercontractility, such as hypertension, cerebral or coronary vasospasm, bronchial asthma, bladder hyperactivity, and erectile dysfunction.

Experimental Section

Chemical synthesis

The synthesis of new LCA lateral chain analogs has been achieved using several synthetic methods (Schemes 1–3). Lower homologues of LCA, that is, C23 acid (**3**) and C23 nitrile (**4**), were prepared by the method of Schteingart and Hofmann.^[40] Treatment of formylated product (**1**) with sodium nitrite in a mixture of trifluoroacetic anhydride with trifluoroacetic acid (second order Beckmann rearrangement) yielded the C23 nitrile derivative (**2**). Controlled basic hydrolysis was performed, which at the same time eliminated the formyl group and gave the desired compounds as a mixture of C23 acid (**3**) and C23 nitrile (**4**). After the workup, both products were separated and characterized. The synthesis of LCA C-23 nitro (**7**) and LCA C-22 aldehyde (**8**) was accomplished as shown in Scheme 1. Formyl protected LCA (**1**) was subjected to a modified Hunsdiecker reaction (iododecarboxylation) in the presence of $\text{pb}(\text{OAc})_4/\text{hv}$ to yield the corresponding formylated 23-iodo derivative (**5**). Purification of compound **5** was achieved by crystallizing it with ethyl acetate without going through the tedious column separation. The cleavage of the formyl group was achieved by stirring compound **5** with K_2CO_3 in ethanol at room temperature to obtain 3 α -hydroxy-23-iodo derivative (**6**), which was used directly to the next step without further purification. Compound **6** was reacted with silver nitrite in dimethyl sulfoxide (DMSO) at room temperature. This reaction gave the two products 23-nitro (**7**) and 22-aldehyde (**8**) (carbonyl at C23) in nearly equal amounts.

Dehydration of primary amide **9** with phosphorus oxychloride in pyridine rendered the 3 α -hydroxy-23-cyano analog (**10**).^[41] Compound **10** was stirred with *p*-toluene sulfonic acid in methanol at room temperature for three days to procure the de-acetylated derivative (**11**).^[42] The desired 3 α -hydroxy-23-tetrazole (**12**) was conveniently prepared in good yield by refluxing the nitrile derivative (**11**) with sodium azide, and triethylamine hydrochloride in toluene (Scheme 2).

Scheme 3 shows the synthesis of several final products using the sulfonate intermediate (**13**). Synthesis of LCA 3 α -hydroxy-24-tetrazole (**16**) was achieved in straightforward two-step procedures from the tosylate (**13**). The tosylate (**13**) was reacted with sodium cyanide in DMSO at 90°C to obtain the cyano analog (**14**), which was then refluxed with sodium azide, and triethyl amine hydrochloride in toluene to render the desired tetrazole (**16**). The higher homologue acid (**15**, carboxyl group at C25) was easily prepared by treating intermediate (**14**) with sodium hydroxide in ethanol. In an attempt to obtain 3 α -hydroxy-C24-sulfonate (**18**), we utilized the versatile intermediate tosylate (**13**). The tosylate **13** was refluxed with sodium iodide in acetone to get the iodo analog (**17**), which was then refluxed with sodium sulfite in aqueous ethanol and yielded the required 3 α -hydroxy-24-sulfonate (**18**).^[43–44] The 3 α -hydroxy-24-nitro derivative (**19**) was prepared by reaction the iodo analog (**17**) with silver nitrite in DMSO. This reaction yielded the 23-aldehyde (**20**) (carbonyl at C24) in nearly equal amounts as a bi product.

The C24- amine (**21**), C24- amide (carbonyl at C24) (**22**) and a diol (**23**) were prepared according to literature procedures.^[45–46]

All chemicals and solvents were purchased from Aldrich and used without further purification. Moisture sensitive reactions were performed under a nitrogen atmosphere. TLC was used to monitor the progress of all reactions on silica gel plates (Analtech, Inc.). Fisher scientific Da visil grade 1740 (170–400 mesh) was used for flash chromatography to purify some of the LAC analogs. ^1H NMR spectra were recorded on a Bruker AR, 300-MHz spectrometer. Chemical shifts were expressed in δ values (ppm) referenced to the TMS and coupling constants (J values) in Hz. Mass spectral data were determined on a Bruker-HP Esquire-LC spectrometer (ESI-MS). Elemental analysis (C, H, N) was performed by Atlantic Microlab, Inc. (Norcross, GA), and results were within $\pm 0.4\%$ of the theoretical values for the formula provided.

Detailed synthetic protocols, ^1H NMR, Mass spectral data and results of elemental analysis are available as Supporting Information.

cRNA preparation and injection into *Xenopus laevis* oocytes

Cbv1 (AY330293) and $\beta 1$ cRNA preparation was performed as described in detail elsewhere.^[24,47] Briefly, cRNA was dissolved in diethyl polycarbonate-treated water at 10 (cbv1) and 30 ($\beta 1$) ng/ μL ; 1- μL aliquots were stored at -70°C . Oocytes were removed from *Xenopus laevis* (Xenopus Express), cRNA-injected, and prepared for patch-clamping as described.^[48–49] The care of animals and animal protocols were performed in accordance to the guidelines of the Animal Care and Use Committee of the University of Tennessee Health Science Center, which is an Association for Assessment and Accreditation of Laboratory Animal Care-accredited institution (#A3325-01). The interval between cRNA cytosolic injection and patch-clamping was 36–48 h.

Electrophysiology data acquisition and analysis

Ionic currents were recorded from excised, inside-out patches. Bath and electrode solutions contained 130 mM Kgluconate, 5 mM EGTA, 1.6 mM HEDTA, 2.28 mM MgCl_2 ($[\text{Mg}^{2+}]_{\text{free}} = 1 \text{ mM}$), 15 mM HEPES, 5.22 mM CaCl_2 ($[\text{Ca}^{2+}]_{\text{free}} = 10 \mu\text{M}$), pH 7.35. Free Ca^{2+} was calculated with MaxChelator Sliders (Stanford University) and validated experimentally using Ca^{2+} -selective and reference electrodes (Corning). Patch-recording electrodes were made as described previously.^[48] An agar bridge with gluconate as the main anion was used as ground electrode. Solutions were applied onto the cytosolic side of excised patches using an automated, pressurized DAD12 system (ALA) through a micropipette tip with an internal diameter of 100 μm . Experiments were carried out at room temperature ($20\text{--}22^\circ\text{C}$). Ionic currents at single channel resolution were recorded using an EPC8 amplifier (HEKA) at 1 kHz. Data were digitized at 5 kHz using a Digidata 1320A A/D converter and pCLAMP 8.0 (Molecular Devices). As index of channel steady-state activity, we used the product of the number of channels in the patch (N) and the channel open probability (P_o). NPo was obtained using a built-in option in Clampfit 9.2 (Molecular Devices) from 30 s of gap-free recording under each condition. Plotting, fitting, and statistical analysis of the data were conducted using Origin 7.0 (Originlab) and InStat 3.0 (GraphPad). Statistical analysis was conducted using one-way ANOVA and Bonferroni multiple comparison test; significance was set at $P < 0.05$. Data are expressed as mean $\pm$ SEM; n = number of patches (each patch was obtained from a different oocyte).

Chemicals and compound application

5 β -cholanic acid-3 α -ol (LCA) was purchased from Steraloids. All other chemicals were synthesized as described above (see Synthetic Protocols). On the day of the experiment, a stock solution (333 mM) of LCA and/or related analogue was freshly made in DMSO by sonication for 5 min. Steroid-containing stock solution was diluted 1/10 in 95% ethanol and further diluted with bath solution to final cholane concentration. The DMSO/ethanol vehicle in bath solution corresponding to a given cholane concentration was used as control perfusion. Maximum DMSO/ethanol final concentrations corresponding to 300 μ M cholane reached 0.10/0.90%.

Computational modeling of cholane–BK β 1 complex

Computational modeling of LCA (non-ionized form) structure was performed as described.^[24] BK β 1 protein sequence (FJ154955) extending from BK β 1 residue L157 to V178, which corresponds to the BK β 1 transmembrane domain 2 (TM2),^[50] was modeled as an ideal α -helix and optimized with the AMBER99 force field to a root mean square gradient (RMSG) of 0.1 kcal $\diamond$ mol⁻¹ $\diamond$ $\text{\AA}^{-1}$.^[51] The dielectric constant for the model was set at 3, a value that is appropriate for the membrane lipid interior.^[52] LCA was manually placed onto the proposed docking site in BK β 1 TM2, formed by T169, L172, L173. Then, the LCA-BK β 1 complex was optimized with computational energy minimization routine using the MM94 force field.^[53] Finally, molecular dynamics (MD) simulation was performed using MM94 force field. MD simulation was run for 500 ps using 1-ps steps. Starting LCA-BK β 1 complex was heated from 0 to 300 °K within the first 100 ps of the simulation, with stability and structural changes of the complex over time being assessed throughout the 101–500 ps (production phase). A resulting complex is displayed in Figure 1a. All procedures were performed using MOE 2010.10 (Chemical Computing Group).

Computational prediction of functional group overall volume and net charge

The overall volumes and partial charges were calculated using Schrodinger 2011 molecular modeling suite (Schrodinger Inc.). Partial volumes ($\text{\AA}^3$) for each of the functional groups were calculated by subtracting the volume of the common steroidal moiety from the total volume for each complete molecule. QikProp module in the Schrodinger software was used for volume calculations. To calculate the partial charges, each molecule was optimized for its geometry using Macromodel module with OPLS_2005 (Optimized Potentials for Liquid Simulations) forcefield. Charges from each atom in the functional groups were added together to obtain the total partial charge for the group.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Cholane chemical structure and interaction with BK β 1 transmembrane (TM) domain

a) Chemical structure of cholane steroids presents several distinctive features. “R” defines the portion of lateral chain modified in our current analogue series. **b)** Proposed model for the BK β 1 TM2 domain-cholane interaction includes hydrogen bonding between cholane hydroxyl group at C3 and T169, and hydrophobic interactions between A/B steroid rings and L172, L173. Aforementioned amino acids are highlighted. Computational modeling places A176 and K179 in the close vicinity of cholane molecule docked on BK β 1 subunit

TM2 domain. However, nonconservative substitutions of these amino acids do not ablate cholane sensitivity of BK channel.

Figure 2. Modifications of lateral chain length significantly decrease cholane-induced BK channel activation

a) Compounds with various lateral chain length. b) Original records of BK currents at single-channel resolution depicting activity of the channel before, during, and after application of 150 μM LCA. Channel openings are shown as downward deflections; arrows indicate baseline (channel closed). $V_m = -40$ mV, $[\text{Ca}^{2+}]_i = 10$ μM . c) Averaged increase in BK channel steady-state activity (NPo) evoked by LCA analogies. Dashed line represents lack of effect. *Significantly different from LCA-induced increase in BK channel (NPo) ($P < 0.05$). d) Concentration response curves (CRCs) for LCA (O, $\text{EC}_{50} = 44.0$ μM) and compound 15 (Δ , $\text{EC}_{50} = 42.8$ μM). Each point represents the average of no less than 3 patches, each patch obtained from a different oocyte. Each data point on the graph in this figure and Figure 4c was obtained from: Normalized effect = $(\text{NPo}_{\text{Compound at any given concentration}} / \text{NPo}_{\text{Matching control}}) / (\text{NPo}_{\text{Compound at Emax}} / \text{NPo}_{\text{Matching control}})$.

Figure 3. Modifications at C25 render compounds with lesser ability to activate BK channel when compared to LCA

a) Compounds with modifications at C25. b) Averaged increase in BK channel steady-state activity (NPo) evoked by LCA analogues. Dotted line represents lack of effect.

*Significantly different from LCA-induced increase in BK channel NPo (P<0.05).

Figure 4. Modifications of functional group carried by lateral chain reveals BK channel activators

a) Compounds with various functional group substitutions in lateral chain. b) Averaged increase in BK channel steady-state activity (NPo) evoked by LCA analogies. Dashed line represents lack of effect. *Significantly different from LCA-induced increase in BK channel NPo ($P < 0.05$). c) Concentration response curves (CRCs) for LCA (○, $EC_{50} = 44.0 \mu\text{M}$), compounds **7** (▽, $EC_{50} = 52.8 \mu\text{M}$), and **11** (△, $EC_{50} = 46.4 \mu\text{M}$). Each point represents the average of no less than 3 patches, each patch obtained from a different oocyte. Data are normalized to the maximum effect detected for each compound according to formula in legend to Figure 2.

scheme 1. Synthesis of LCA-derived nitro (**7**) and aldehyde (**8**) in the lateral chain.

scheme 2.

Synthesis of LCA-derived tetrazole (12) in the lateral chain.

scheme 3. Synthesis of several final products using the sulfonate intermediate (**13**).

Table 1

Functional group overall volume and net charge.

Compound	Functional group	Overall volume (Å ³)	Partial charge at pH=7 (e)
LCA, 3, 15	–COOH	92	–0.90
21	–NH ₂	38	+0.69
12, 16	–CN ₄ H	157	–0.98
7, 19	–NO ₂	78	–0.20
11, 14	–CN	65	–0.10
18	–SO ₃ H	136	–0.84
13	–O–SO ₂ –C ₆ H ₄ –CH ₃	438	N/A ^[a]
22	–CO–NH ₂	102	N/A ^[a]
17	–CH ₂ I	135	N/A ^[a]
23	–CH ₂ OH	92	N/A ^[a]

^[a]: N/A, not applicable due to the lack of charge at physiological conditions.