Molecular mechanism of pharmacological activation of BK channels

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Large-conductance voltage- and Ca2+-activated K+ (Slo1 BK) channels serve numerous cellular functions, and their dysregulation is implicated in various diseases. Drugs activating BK channels therefore bear substantial therapeutic potential, but their development has been hindered in part because of the mode of action remains obscure. Here we provide mechanistic insight into how the dehydrobiotic acid derivative Cym04 activates BK channels. As a representative of NS1619-like BK openers, Cym04 reversibly left-shifts the half-activation voltage of Slo1 BK channels. Using an established allosteric BK gating model, the Cym04 effect can be simulated by a shift of the voltage sensor and the ion conduction gate equilibria toward the activated and open state, respectively. BK activation by Cym04 occurs in a splice variant-specific manner; it does not occur in such Slo1 BK channels using an alternative neuronal exon 9, which codes for the linker connecting the transmembrane segment S6 and the cytosolic RCK1 domain—the S6/RCK linker. In addition, Cym04 does not affect Slo1 BK channels with a two-residue deletion within this linker. Mutagenesis and model-based gating analysis revealed that BK openers, such as Cym04 and NS1619 but not mallotoxin, activate BK channels by functionally interacting with the S6/RCK linker, mimicking site-specific shortening of this purported passive spring, which transmits force from the cytosolic gating ring structure to open the channel's gate.

K+ channel opener | patch clamp | KCNMA1 | Kcα1.1

Large-conductance voltage- and Ca2+-activated K+ channels (BK channels) serve numerous but specific physiological roles in virtually every cell type (1), for example, regulation of shape and frequency of action potentials in select neurons (2) and control of the tone of vascular smooth muscle cells (3). This functional versatility of BK channel proteins is conferred by a variety of means, including coassembly with auxiliary subunits (4) and extensive alternative splicing (5) of the pore-forming α-subunit encoded by the single gene slo1 (Slo1 gene, Kcα1.1, KCNMA1) (6).

Dysregulation of BK channels has been implicated in a wide spectrum of diseases, including hypertension, urinary incontinence, erectile dysfunction, and epilepsy (1, 7). BK channels are considered promising targets for the treatment of diseases of cellular hyperexcitability, such as excitotoxicity after ischemic stroke (8). An array of synthetic and natural compounds that activate BK channels, referred to as BK openers, has been reported (7), among them synthetic benzimidazolone derivatives [NS004 (9) and NS1619 (10)], biaryl amines [flufenamic acid (11)], biarylureas [NS1608 (12)], biarylthioureas [NS11021 (13)], aryloxindoles [BMS-204352 (8)], arylypyrroles [NS8 (14)], and indole-3-carboxylic acid esters [CGS-7184 and CGS-7181 (15)]. However, the therapeutic deployment of BK openers has been limited in part by the realization that ectopically enhanced activation of BK channels could potentially induce epilepsy and paroxysmal movement disorder (16) and also by the paucity of information about the biophysical and molecular mode of action. The availability of such information is expected to facilitate development of therapeutically useful condition-specific BK openers. For example, it may be desirable to develop agents that specifically open BK channels only when cells are at rest, when depolarized, and/or only when their Ca2+ levels are high.

Activation of BK channels involves allosteric interactions among the pore domain comprising the ion conduction gate (17), the voltage sensor domains, and the intracellular Ca2+-sensing gating ring. Binding of Ca2+ to the cytoplasmic (regulator of conductance for K+) RCK1/RCK2 domains (18, 19), collectively forming the gating ring (20, 21), is thought to cause structural rearrangements, which are then transmitted to the pore domain (17). Activation of the voltage-sensor domains composed of the transmembrane segments S1 through S4 by depolarization or Ca2+ increases probability that the ion conduction gate opens (22, 23). Structurally critical in this allosteric activation is the short stretch of amino acid residues connecting the transmembrane segment S6 and the cytosolic RCK1 domain; this S6/RCK linker is speculated to be important in both Ca2+-triggered and voltage-sensor-initiated opening of the ion conduction gate located in the pore domain (17, 22, 24). In particular, shortening of the S6/RCK linker enhances the channel activity by left-shifting the voltage dependence of the channel, suggesting that the linker may function as a passive spring to regulate the ion conduction gate (25).

Any of the aforementioned allosteric components in the gating machinery of BK channels are potential effectors of BK openers. However, even for the benzimidazolone NS1619 (7, 10), the most widely used BK opener in research settings, it is not clear which of the allosteric components are altered to increase open probability. Cym04 is a dehydrobiotic acid BK opener, distinct from the benzimidazolone family of BK openers (Fig. L4, Left). A previous study suggested that Cym04 might be more potent than NS1619 and represents a suitable scaffold for creating tissue- and function-specific BK openers (26). The development of such reagents requires an elucidation of the biophysical mechanism and an identification of the molecular domain of the channel essential for the Cym04 action. Here we provide a quantitative and mechanistic insight into the action of Cym04 on BK channels. Further, using two alternatively spliced variants expressed in a tissue- and development-specific manner (24) we show that the S6/RCK linker segment plays a critical role in determining the sensitivity of the channel to Cym04.
**Results**

**Cym04 Opens Slo1 BK Channels in a Splice Variant-Specific Manner.** The structural element linking the channel’s pore domain and the cytosolic domain, the S6/RCK linker, plays an important role in allosteric gating of the Slo1 BK channel (17, 24, 25). This linker segment is commonly encoded by exon 9 of the human Slo1 gene. However, we recently identified a splice variant of slo1 using a mutually exclusive alternative exon 9. Slo1 BK channels with such alternative exon 9, here referred to as Slo1_9a, are primarily found in neuronal tissue and display an altered dependence on the membrane potential and the [Ca^{2+}]_{i} (24), consistent with the postulated importance of the S6/RCK linker in the allosteric gating mechanism (25). Because the BK opener Cym04 likely alters the gating machinery, we compared how Cym04 affected channels with the common S6/RCK linker (Slo1) or the distinct linker coded by the alternative exon 9 (Slo1_9a). Slo1 or Slo1_9a channels were expressed in HEK 293 cells, and modulation by Cym04 was analyzed in the inside-out configuration of the patch-clamp method. Experiments were initially performed in the virtual absence of Ca^{2+} to simplify channel gating.

Current responses of both Slo1 isoforms to 10-ms depolarizations are compared in Fig. 1A before and after application of 10 µM Cym04 to the cytosolic side. At the voltages at which channels are activated to <5%, Cym04 rapidly increased the Slo1 current amplitude by approximately fivefold in a reversible manner and did not require previous channel opening (Fig. S1). The increase in Slo1 current size was not accompanied by a noticeable change in the single-channel current size (Fig. 2B); single-channel conductance of Slo1 channels in the range of −100 to 50 mV was unaffected by 10 µM Cym04: 240 ± 3 pS (control) and 244 ± 4 pS (Cym04, n = 12, P = 0.39). The current increase is thus most probably caused by changes in gating of Slo1 by Cym04. Analysis of tail currents indeed showed that Cym04 markedly shifted the half-activation voltage (V_{0.5}) estimated using a modified Boltzmann equation (Eq. 1). Cym04 (10 µM) shifted V_{0.5} of Slo1 channels from 213 ± 4 mV to 184 ± 7 mV (P < 0.001, n = 12). The slope of the curve was unaffected; V_{∞} values were 35.1 ± 1.1 mV (control) and 36.3 ± 0.7 mV (P = 0.21).

In contrast with the significant current increase observed with Slo1, Cym04 failed to alter currents through Slo1_9a even when examined over a wide voltage range (Fig. 1A, Right). The V_{0.5} values in Slo1_9a were 104 ± 2 mV and 107 ± 2 mV before and after application of Cym04, respectively (P = 0.20, n = 7). Cym04 therefore is a BK opener that clearly discriminates between the two splice variants that differ in the S6/RCK linker region. We examined whether other BK openers share the specificity of Cym04 for the Slo1 isoform over Slo1_9a. NS1619 is a well-known BK opener (10) but belongs to a structural class distinct from that of Cym04 (Fig. 1B, Left). We chose 30 µM NS1619 to obtain a shift in V_{0.5} comparable to that obtained with 10 µM Cym04 in Slo1 (ΔV_{0.5} = −36.0 ± 2.6 mV, n = 7; for 10 µM NS1619: ΔV_{0.5} = −21.6 ± 3.9 mV, n = 5). At this concentration, frequently used for in vitro tissue studies, NS1619 did not activate Slo1_9a channels (Fig. 1B, Right). The preferred action of both Cym04 and NS1619 on Slo1 over Slo1_9a suggests that, despite the structural differences, the two BK openers may activate the channels via a common mechanism hinging on the S6/RCK linker. Unlike with the splice variant-specific action of Cym04 and NS1619, mallotoxin (MTx, 2.5 µM), a naturally occurring BK opener in a distinct structural family (27), activated both Slo1 and Slo1_9a by causing a similar large shift in V_{0.5} (ΔV_{0.5} = −38.1 ± 14.7 mV, n = 4 in Slo1 vs. ΔV_{0.5} = −29.0 ± 11.7 mV, n = 5 in Slo1_9a). Slo1 and Slo1_9a are a promising pair of BK channels suited to study the molecular mechanism and isoform specificity of BK activation by Cym04 and NS1619-like BK openers.

**Characterization of Slo1 BK Channel Activation by Cym04.** Changes in electrophysiological characteristics of Slo1 channels, whether by mutations or signaling molecules, can be interpreted using the established gating model of Slo1 (*HA model*; SI Results, and SI Materials and Methods) to gain biophysical and molecular insight (28). Using the HA model as the conceptual framework, we performed an analysis of the changes in Slo1 ionic currents caused by 10 µM Cym04. The concentration dependence of Slo1 channel activation by Cym04 as measured by the shift in V_{0.5} showed that the half-maximal activation was obtained at 5.9 µM, with a Hill coefficient of 1.6 (Fig. S2). Thus, 10 µM Cym04 represents a near-saturating concentration for Slo1 and was used in most of our experiments. The electrophysiological measurements...
currents from inside-out patches measured in the presence of 100 μM Cym04 (squares) or 10 mM Mg\(^{2+}\) (circles) before (open symbols) and after (red filled symbols) application of 10 μM Cym04. Data points are mean ± SEM (n = 6), and continuous curves are data fits according to Eq. 1. Cym04-induced shifts in half-maximal activation voltage (ΔV\(_{0.5}\)) and slope factor (ΔV\(_{s}\)) for experiments as shown in D at different [Ca\(^{2+}\)] and [Mg\(^{2+}\)], as well as in the absence of divalent cations for Slo1 coexpressed with Slo β1 subunit and for BK channels in LNCaP cells. Vertical red lines indicate control values for Slo1 and Slo1_9a.

showed that Cym04 had the following two primary effects on Slo1 currents in the virtual absence of Ca\(^{2+}\). First, Cym04 decelerated deactivation at very negative voltages by ≈2.5-fold but had no effect on activation at very positive voltages (Fig. 2A and Fig. S3). Second, the effectiveness of Cym04 on Slo1 diminished but did not completely disappear at weaker depolarization (Fig. 2B and Fig. S4). For example, at 50 mV, Cym04 induced a 27 ± 11-fold increase in open probability, compared with the 7 ± 3-fold increase found at −50 mV (P < 0.05, n = 6). These observations and others, as well as our simulations, showed that the two main effectors of Cym04 are the channel’s voltage-sensing mechanism and the ion conduction gate (Fig. S4), stabilized by ≈3.0 kJ/mol and ≈1.0 kJ/mol, respectively. Because Cym04 did not significantly alter Slo1 gating currents (Fig. 2C), an impact of the channel opener on the coupling of sensor and gate is suggested.

Our analysis of the results obtained in the virtual absence of Ca\(^{2+}\) suggests that the activating effect of Cym04 may be independent of the Ca\(^{2+}\)- and Mg\(^{2+}\)-dependent activation pathways of the BK channel (29–32). Consistent with this prediction, with a functionally saturating concentration of cytosolic Ca\(^{2+}\) (100 μM) or Mg\(^{2+}\) (10 mM), Cym04 (10 μM) remained effective in shifting the voltage dependence to the negative direction (Fig. 2 D and E), indicating that the channel opener does not directly act as a mimic of these divalent cations. At intermediate [Ca\(^{2+}\)]\(i\), Cym04 remained effective in activating BK channels; there was a trend for a decrease in ΔV\(_{0.5}\) (Fig. 2E), which might be indicative for an increase in the apparent Ca\(^{2+}\) binding constant, but the results were statistically insignificant.

The action of Cym04 was also independent of the presence of the auxiliary subunit β1 (33). In hSlo1+β1 channels, Cym04 shifted V\(_{0.5}\) by −40.1 ± 4.9 mV (n = 6), which is not significantly greater than that with Slo1 alone without β1 (Fig. 2E). Likewise, coassembly with LRRC26, an auxiliary protein conferring a substantial (>100 mV) left-shift of V\(_{0.5}\), as for BK channels in LNCaP cells (34), was without effect: in the virtual absence of intracellular Ca\(^{2+}\), Cym04 shifted LNCaP whole-cell BK channel activation by −32.5 ± 1.9 mV (n = 6) (Fig. 2E). In summary, Cym04 is a reversible BK channel opener that affects channel gating without any requirement for intracellular divalent cations or the presence of accessory subunits.

**S6/RCK Linker Is a Molecular Determinant for BK Channel Activation by Cym04.** The striking difference in Cym04 response between Slo1 and Slo1_9a channels (Fig. 1) served as a starting point for the identification of molecular determinants required for the BK opener action. The two variants differ in 13 nonconsecutive amino acid residues, ranging from the transmembrane segment S6 in the pore domain to the linker to the RCK1 domain (S6/RCK linker) (Fig. 3 A and B). To reveal which of these 13 residues are important for Cym04 action on BK channels, we generated chimeras in the background of Slo1 channels, dividing the area of interest into three regions: the distal part of S6 (which is typically considered to end at I326), a proximal linker segment, and a distal linker segment. Thus, Slo1, in which Cym04 causes a ΔV\(_{0.5}\) of −29 mV, is represented here as “999,” whereas Cym04-insensitive Slo1_9a is represented as “aaa” because the three segments are coded by alternative exon 9. Chimeras were tested to identify which substitution impairs the Cym04-induced shifts in V\(_{0.5}\). Although ΔV\(_{0.5}\) of chimera a99 with the alternative S6 segment was not significantly different (P = 0.12) from that of Slo1 (“999”) channels, the shift was significantly smaller for chimera 9a9 (P < 0.005), indicating that the proximal and/or channels in the absence of divalent cations. Data points are mean ± SEM, n indicated in parentheses. *P < 0.05; **P < 0.01, comparing with Slo1 in zero Ca\(^{2+}\).
found in Slo1 to Slo1_9a revealed that the mutations G327L, K330N, and Y332F, but not N328S, significantly diminished—but not completely abolished—the left-shift in $\Delta V_{0.5}$ by Cym04.

The particularly diminished effect of Cym04 in K330N (Fig. 3C) led us to examine whether substitutions of other amino acids at this position also impaired the activating effect of Cym04. Neither the charge reversal mutation K330E nor the charge neutralization mutation K330A diminished the left-shift in $V_{0.5}$ by Cym04 (Fig. 3C).

Many of the channel mutants with diminished sensitivity to Cym04 identified above including Slo1_9a exhibited a left-shifted $V_{0.5}$ under control conditions (Fig. 3C, Left). Because Cym04 causes a left shift in the voltage dependence, the diminished effectiveness of Cym04 in these mutants may be a universal feature associated with any left-shifted mutant. We therefore tested whether other mutations known to induce a left shift in BK channel gating also attenuated BK channel activation by Cym04. Four single-site mutations—M442A in the cytosolic RCK1 intersubunit interface, G733D in the RCK2 intrasubunit interface, F315Y in the transmembrane segment S6, and R207G in the transmembrane segment S4—induced shifts in $V_{0.5}$ by approximately $-50$ mV under control conditions but failed to attenuate the Cym04 effect (Fig. 4B). Because of the slow activation time course of mutant F315Y, the use of 10-ms depolarizations will overestimate $V_{0.5}$ values; however, even under strongly extended pulse protocols Cym04 still left-shifted $V_{0.5}$ (Fig. S5). Therefore, the diminished effectiveness of Cym04 is not a common feature of every left-shifted mutant.

The S6/RCK linker has also been implicated to play a pivotal role in voltage-dependent activation of BK channels, in particular because shortening the linker left-shifts the voltage dependence (25). Therefore, Cym04 may trigger the same conformational change induced by shortening of the S6/RCK linker. This postulate in turn suggests that shortening of the linker renders Cym04 less effective. We found that a deletion of two amino acids, Y336 and either S335 or S337, in the distal linker segment of Slo1 (Fig. 3B; distal linkers are crucial for Cym04 action on Slo1 channels. The high-impact area was further narrowed down to the proximal linker part because chimera 9a9 ($P < 0.005$) but not 99a ($P = 0.63$) exhibited attenuated Cym04-induced activation. Within the proximal linker segment, Slo1 and Slo1_9a differ at four amino acid positions (327, 328, 330, and 332) (Fig. 3B). The results from the single point mutations changing each position from that...
“LL−2” in Fig. 4) shifted the voltage dependence of activation by −33 mV under control conditions and diminished BK channel activation by Cym04 (Fig. 4). In contrast, an insertion of two alamines between S337 and A338 in Slo1 in the distal linker segment (Fig. 3B; “LL+2” in Fig. 4) caused a 39-mV right shift of V0.5 under control conditions but did not affect BK activation by Cym04 (Fig. 4B).

The reduced potency of Cym04 in mutant LL−2 may either result from a reduction in linker length per se or from structural changes at the deleted amino acid positions. The first possibility seems unlikely because alternative double-residue deletions at neighboring sites did not reduce the left-shift in V0.5 upon Cym04 application (Fig. S6). Single-site mutations at positions S335, Y336, and S337 did not noticeably diminish the impact of Cym04 with the exception of Y336F (Fig. S6). Moreover, mutations Y336G, -D, and -E strongly impaired functional channel expression.

Discussion

Because activation of BK channels typically reduces cellular excitability, BK openers hold promise to treat a wide variety of disease, such as stroke, urinary incontinence, asthma, and arterial hypertension (7). However, no BK opener has been approved yet for clinical use, and clinical trials for several compounds such as BMS-204352 have been discontinued (7). The major obstacles to development of the therapeutic BK openers include the lack of a firm mechanistic understanding of how BK openers work at a molecular level and the absence of a rationale for selecting best-suited openers among the diverse classes of BK-acting drugs. To facilitate rational drug design, a systematic classification of BK openers based on their molecular modes of action, including the identification of binding sites and structural scaffolds mediating their activity, is urgently needed.

Here we have provided in-depth study on the molecular mode of BK activation by Cym04, a dehydroabietic acid derivative, previously reported (26) to activate BK channels more potently than the prototypical BK opener, NS1619. Cym04 shifts the voltage dependence of Slo1 BK channels by up to 40 mV to the negative direction. This shift is less than the maximum shifts reported for dehydrosoyasaponin-1 (DHS-1) (35), MTx (27), or NS1619 (10), but it should be large enough to modulate physiological responses; in comparison, phosphorylation of BK channels by physiologically relevant protein kinases A and G causes a 15–35 mV left shift (36).

Detailed electrophysiological analysis using the established allosteric model of BK channel gating (“HA model”) showed that the dehydroabietic acid Cym04 has two primary effects on the channel gating. Firstly, Cym04 favors the voltage-dependent activated channel state described by the equilibrium constant J and the open conformation of the ion conduction gate characterized by the equilibrium constant L. These two changes were also observed for the benzimidazolone NS1619 (Fig. S7), a widely used BK opener in research settings. Thus, although structurally distinct, Cym04 and NS1619 belong to the same functional class. On the basis of the functional targets according to the HA gating model, Cym04 and NS1619 may be called JLN+ modulators. This classification may be useful for selecting experimentally or therapeutically appropriate BK openers. For example, if resting vascular tone is to be selectively decreased, LN+ modulators, which would open BK channels without any requirement for depolarization or Ca2+, may be desired. Although it is not clear which of the HA model parameters are affected, BMS-204352 (8), a previously described BK opener, may belong to this class. In contrast, JN+ modulators facilitating the voltage sensor activation may be more appropriate to specifically counteract depolarization.

Our work points to the importance of the S6/RCK linker segment in conferring the sensitivity to Cym04, which is without effect on Slo1_9a with a distinct S6/RCK linker sequence. This ~17-residue stretch has been previously suggested to work as a passive mechanical spring; the shorter the linker, the more left-shifted the voltage dependence of activation without a noticeable change in steepness (25). However, our data show that the location where the linker is truncated is an important factor. The finding that a S6/RCK deletion mutation (“LL−2”; Fig. 4) diminishes the shift in voltage dependence by Cym04 may be interpreted to suggest that binding of Cym04 to Slo1 induces a structural change similar to that observed when the S6/RCK linker is shortened at this particular position.

Whether the S6/RCK linker itself represents a binding site for Cym04 is not certain. The possibility that Cym04 binds to the S6/RCK linker, which is already implicated in regulation of the voltage dependence, is attractive. A previous structure–activity study of Cym04 using the peak current size at one voltage as the dependent variable suggested that the carboxylic acid moiety and the hydrophobicity of the benzene ring of Cym04 are functionally important (37). Alternatively, the S6/RCK linker may be a coupling component functionally connecting the binding site for Cym04 located elsewhere to the effector, namely the voltage sensors and the main ion conduction gate. However, another possibility is these changes in the S6/RCK linker sequence may hinder the access of Cym04 to its binding site located elsewhere.

The splice variant-specific action of Cym04 and NS1619 preferring Slo1 to Slo1_9a described here has both experimental and therapeutic implications. NS1619 is often used to infer the involvement of BK channels in a variety of physiological phenomena; the absence of a functional impact of NS1619 is typically taken as evidence against the involvement of BK channels. However, this line of reasoning must be reevaluated in light of our finding that NS1619 and Cym04 have no effect on Slo1_9a, which is found primarily in the nervous system (24). Although the relative expression level of Slo1_9a may be less than that of Slo1 (24), the presence of Slo1_9a could make a marked impact if Slo1_9a and Slo1 physiologically form NS1619/Cym04-insensitive heteromultimers. In fact, coexpression studies (Fig. S8) suggest that the Slo1/Slo1_9a subunit stoichiometry significantly affects the channel’s susceptibility toward Cym04. Therapeutically, the insensitivity of Slo1_9a and its preferred expression in the nervous system offer a promising opportunity to develop tissue-specific BK modulators based on the dehydroabietic acid scaffold. Development of such tissue-specific therapeutic BK openers is clearly needed. Although activation of vascular smooth muscle BK channels may lead to beneficial consequences, genetic studies suggest that overactivation of neuronal BK channels may cause epilepsy and paroxysmal movement disorder (16). However, subtype-specific BK openers may be beneficial in forms of epilepsy that are characterized by an impaired BK function (38). Cym04 and NS1619, which preferentially act on Slo1 to Slo1_9a, and DHS-1, which up-regulates Slo1+β1 complexes (4, 7) predominantly found in vascular smooth muscle cells (33), may constitute good starting compounds for further drug development.

In summary, our study reveals a mechanism by which the dehydroabietic acid Cym04 activates BK channels. It facilitates activation of the voltage sensor and opening of the ion conduction gate. Using the established allosteric model of the Slo1 BK channel gating, Cym04 may be considered a JLN+ modulator, whereby the J equilibrium not only reflects the state of the voltage sensor but also includes its coupling to the gate. Like the well-known BK opener NS1619, Cym04 selectively activates Slo1 without any effect on the splice variant Slo1_9a, which differs in the S6/RCK linker and is expressed preferentially in the nervous system. Therefore, Cym04 may represent a solid foundation for further tissue- and function-specific BK openers.

Materials and Methods

Chemicals and Solutions. Cym04 was synthesized as described previously (26), dissolved in DMSO at 10–50 mM stock solution, and stored at −20 °C. NS1619 and MTx were obtained from Sigma and stored as 10–20 mM DMSO stock
Cell Culture and Molecular Biology. HEK 293 cells were maintained and transfected with expression plasmids encoding hSlo1 (α) (U11058) and mutants thereof, as described earlier (39). Marker plasmids encoding CDB were cotransfected (17% of total DNA) to allow identification of transfected cells via binding of Dynabeads (Invitrogen). Cloning of the Slo1.9a splice variant was previously described (24). The pC-neo-based (Promega) expression plasmid encoding hSlo1 was modified to facilitate mutagenesis introduction of a silent Pmel restriction site within the hSlo1 coding region and removal of an EcoRI-site within the 3′ UTR. Mutations of SLO1 were introduced via overlap extension PCR mutagenesis and verified by sequencing.

Electrophysiological Recordings. Experiments were performed at room temperature (20–24 °C) using an EPC-10 patch-clamp amplifier controlled via PatchMaster software (HEKA Elektronik). Records were low-pass filtered at 10 kHz (eight-pole Bessel) and sampled at 50–100 kHz. Data analysis was performed using FitMaster (HEKA) and IgorPro (WaveMetrics) software. Patch pipettes from borosilicate filament had tip resistances of 1–3 MΩ for macropatch recordings and approximately 10 MΩ for single-channel recordings. The standard internal solution contained 140 mM KCl, 10 mM EGTA, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4) (KOH). For the 10 mM Mg2+-containing solution, 14.3 mM MgCl2 was added. For solutions containing 3 and 10 mM free Ca2+, 10 mM EGTA was replaced by 10 mM HEDTA plus 5.6 mM and 8.3 mM CaCl2, respectively, as calculated by Patchayer’s Power Tools (http://www.mpibpc.mpg.de/groups/neher/index.php?page=aboutptt). To result in 100 mM free Ca2+, CaCl2 was added without further Ca2+ buffering, assuming 20 mM Ca2+ in the water. The external solution always consisted of (in mM): 140 KCl, 2 CaCl2, 2 MgCl2, and 10 Hepes (pH 7.4) (KOH).

To assay the voltage dependence of BK channel gating, 10-msec depolarizations to various voltages, after a 20-msec prepulse to −150 mV to close the channels, were followed by a constant test pulse to −150 mV. Voltage steps between −110 and −150 mV were used for offline leak subtraction. Instantaneous tail current amplitudes (Itail) at −150 mV were plotted against voltage and fitted with Boltzmann functions as operational data descriptors:

$$\text{ltail} = \frac{I_{\text{max}}}{1 + e^{-(V - V_{1/2})/\sigma}}$$

where \(I_{\text{max}}\) is the maximum \(ltail\) for control conditions, used to normalize \(ltail\) for averaging and to calculate open probabilities (\(P_{\text{open}}\) as shown in Fig. 1). \(V_{1/2}\) is the voltage needed for half-maximal activation, \(V_c\) is a slope factor representing the voltage difference needed for an e-fold activity change, and \(\sigma\) the maximal \(ltail\) obtained over the full voltage range.

Single-channel analysis was performed on current records from patches containing fewer than five channels as determined at positive voltages (≥280 mV) and in the presence of intracellular Ca2+ to obtain maximal open probability. Alternatively, single-channel data were obtained at negative voltages, and the number of channels was calculated from the macroscopic current amplitude at 250 mV assuming a single-channel conductance of 24 pS and an open probability of 0.5. For gating current measurements refer to SI Materials and Methods.

All data are presented as mean ± SEM (n), where n is the number of independent experiments. Statistical differences between groups of data were tested using a two-sided Student t test followed by Holm-Bonferroni correction.

Additional Methods. Details on data analysis in the framework of a Horrigan-Aldrich activation model are provided in SI Materials and Methods.

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Supporting Information

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SI Results

Model-Dependent Analysis of BK Channel Activation by Cym04. Single Boltzmann functions do not very accurately describe the voltage-dependent gating of BK channels. We therefore investigated the mechanism of Slo1 BK channel activation by Cym04 using a well-established allosteric gating model of Horrigan and Aldrich ("HA model") (1). In the absence of Ca\(^{2+}\), this model can be substantially simplified (Fig. S4A). To maximize the experimentally accessible voltage range, \(P_{\text{open}}\) data were compiled from macroscopic and microscopic data. Tail currents at \(-150\) mV after 10-ms depolarizations to voltages \(\geq 100\) mV were used to calculate open probabilities \(P_{\text{open}}\) on the basis of the maximum tail current amplitude obtained in 100 \(\mu\)M Ca\(^{2+}\). The number of channels in each patch was estimated from the current amplitude at 250 mV. From the same patches \(P_{\text{open}}\) was estimated by single-channel analysis at voltages \(\leq 50\) mV (Fig. 2B). At \(+50\) mV BK channel activation by Cym04 was stronger than at \(-50\) mV (27 \pm 11-fold vs. \(7 \pm 3\)-fold; \(P < 0.05, n = 12\)), indicating that Cym04 affects the equilibrium constant \(K\), which describes the main voltage sensor behavior. For a fit of averaged \(P_{\text{open}}\) data with the HA model, two important constraints based on macroscopic data were implemented: (i) because Cym04 did not alter the slope of the macroscopic conductance–voltage data (Fig. 1A and D), \(z_1\) was fixed to 0.69 \(e_0\), as calculated from \(V_z\); (ii) \(z_{J}\) was calculated from the voltage dependence of the macroscopic time constants derived from monoeponential fits in the absence of intracellular Ca\(^{2+}\) at very negative voltages (\(z_{J}\)) and high positive voltages \((z_{P})\) (Fig. S3). \(z_{J}\) determined in this manner was not significantly altered by Cym04. With \(z_{J}\) fixed to the control value of 0.294 \(e_0\), the voltage dependence of \(P_{\text{open}}\) was fit to control data for voltages \(\leq -50\) mV, yielding \(L_0 = 7.8 \pm 2.3 \times 10^{-6}\), the latter was held constant in subsequent fits for the full voltage range to optimize \(V_{HA0.5} = 122 \pm 2\) mV and \(D = 9.14 \pm 0.04\). Best fits to the data upon Cym04 application were obtained for a shift of \(V_{HA0.5} = -45\) mV plus either an increase in \(D\) by 11% or an increase of \(L_0\) by 1.5-fold. The goodness of both fits was almost indistinguishable; however, because Cym04 deaccelerated Slo1 BK channel deactivation kinetics at very negative voltages, we deemed that the latter approach is better justified—a dual effect of Cym04 on \(V_{HA0.5}\) and \(L_0\). This was also in good agreement with our observation that even at \(-125\) mV Cym04 still activated Slo1 BK channels, although significantly weakened (2.1 \pm 0.5-fold) than at \(-50\) mV \((3.5 \pm 0.4\)-fold, \(n = 4\)). Alternative attempts to fit the data, based on changes in the \(L\) equilibrium only, resulted in worse fits (Fig. S4B and C), with more than 10-fold increased mean-square deviation from the voltage dependence of \(P_{\text{open}}\) data. We thus conclude that Cym04 activates Slo1 BK channels by stabilizing (i) the activated state of the voltage sensor \((J\) equilibrium) and (ii) the open conformation of the channel’s pore/gate \((L\) equilibrium). Channel activation by NS1619 occurred qualitatively in a similar fashion; it was approximately eightfold stronger at 50 mV compared with \(-50\) mV, clearly indicating changes in the \(J\) equilibrium (Fig. S7). In the framework of the HA model, Slo1 BK channel activation by NS1619 could be simulated similarly, namely a 35 mV left-shift in \(V_{HA0.5}\) and a 1.7-fold increase in \(L_0\).

SI Materials and Methods

HA Model Data Analysis. In the absence of intracellular Ca\(^{2+}\), the HA model (1) simplifies to a gate equilibrium \((L)\) that can be allosterically affected by the voltage-sensor \((J)\) equilibrium. Both equilibriums are described in a formally identical manner:

\[
L = L_0 \cdot \exp\left(\frac{z_L \cdot V}{k_B \cdot T}\right)
\]  

and

\[
J = J_0 \cdot \exp\left(\frac{z_J \cdot V}{k_B \cdot T}\right)
\]

with \(L_0\) and \(J_0\) being the zero voltage values of \(L\) and \(J\), \(z_L\) and \(z_J\) are the corresponding partial charges, \(k_B\) is the Boltzmann constant, and \(T\) the absolute temperature. \(V_{HA0.5}\) is defined for \(J = 1\),

\[
V_{HA0.5} = -\frac{k_B \cdot T}{z_J} \cdot \ln(J_0).
\]

The open probability \((P_{\text{open}})\) is given by

\[
P_{\text{open}} = 1 / \left(1 + \frac{(1 + J)^{4}}{L \cdot (1 + D \cdot J)^{3}}\right).
\]

For full HA model analysis, \(P_{\text{open}}\) data were initially fitted for voltages \(\leq -50\) mV, where \(P_{\text{open}}\) can be approximated by \(L\), yielding values for \(L_0\) and \(z_L\), which were held constant throughout further model refinements.

Gating Current Measurements. For gating current measurements internal and external K\(^+\) in the recording buffers was replaced with N-methyl-D-glucamine and tetraethylammonium, respectively. In the whole-cell mode, 2-ms depolarizations of up to 300 mV were applied. Capacitive transients were compensated for using a P/6 protocol. Remaining ionic currents were fitted with a second-order exponential function within the last 1.5 ms of the depolarization episode, extrapolated back to the start of depolarization, and subtracted. Gating charge \((Q)\) was obtained by integrating the gating currents within 2 ms; upon normalization to the maximal gating charge, it was plotted against voltage and fitted with a Boltzmann function (Q-V curve).

Fig. S1. Large-conductance voltage- and Ca$^{2+}$-activated K$^+$ (BK) channel activation by Cym04 is reversible and does not require previous channel opening. (A) BK currents in an inside-out patch from a hSlo1-expressing HEK 293 cell were elicited every second by 10-ms depolarizations to 130 mV. Representative current responses are shown under control conditions (Ctrl), for the first depolarization after a 2-min preincubation period with 10 μM Cym04 present (+Cym04 #1), and 2 min after washout (Wash #1). During a second application to the same patch the current amplitude was further increased (+Cym04 #2) and almost approached the control level after washout (Wash #2). (B) Maximal outward current amplitudes as a function of time, with green circles indicating the time points corresponding to the current traces shown in A. At 60 s, a control wash was performed. Red arrows indicate Cym04 application. Two microliters of a 10-mM stock solution were added to the 2-mL bath and intensively mixed, resulting in an effective exchange time of less than 2 s. For washout the bath solution was replaced with compound-free solution resulting in an approximately 1000-fold reduction in compound concentration within ≈15 s. For the second Cym04 application, the onset of BK channel activation was analyzed with a monoexponential fit (red curve), here yielding a time constant of 3.5 s. (C) Current amplitudes were normalized to the control current before Cym04 application. To test for a possible state or time dependence, mean data (n = 4, each) are compared for different conditions: RD (repeated depolarization): BK activation was monitored directly upon Cym04 activation via repeated (1 Hz) depolarizations and quantified after 60 s. No RD: patches were preincubated for 2 min at −100 mV in Cym04-containing bath solution, before repeated depolarizations (1 Hz) restarted, with the relative amplitudes shown for the first and the 60th pulse, indicating no further time dependence.

Fig. S2. Concentration dependence of Cym04. Concentration dependence of shift in half-maximal voltage ($\Delta V_{0.5}$) by Cym04. The smooth curve is the result of a Hill fit (see text).
Fig. S3. Cym04 affects macroscopic BK current kinetics. (A and B) Representative current traces before (Left) and after application of 10 μM Cym04 (Right) elicited by the voltage protocol shown on top. Monoexponential functions were used to fit the kinetics of channel activation (A) or deactivation (B) at various voltages. Fit curves are overlaid in blue (Left, Control) and red (Right, Cym04). (C) Mean time constants ± SEM (n = 6–7) as a function of voltage. At extreme voltages data were fit (blue, Control; red, Cym04) with the function $\tau = \tau_0 \exp \left(\frac{z_N e V}{k_B T}\right)$, yielding the partial valence for the voltage dependence of the time constant at very negative voltage ($z_N$) and very positive voltage ($-z_P$), respectively, with $z_L = z_N + z_P$. Cym04 did not significantly alter the partial valences of 0.159 ($z_N$) and 0.135 ($z_P$).
Constrained HA-model analysis of Slo1 BK channel activation by Cym04. (A) HA equilibrium model used for quantitative analysis of allosteric BK channel gating. The ion conduction gate is either closed (C) or open (O) and described by the equilibrium constant $L$. Each of the four voltage sensors is at rest (R) or activated (A) and described by the equilibrium constant $J$. The Ca$^{2+}$ sensors of each of the four subunits may be empty (X) or Ca$^{2+}$ bound (XCa$^{2+}$), and the constant $K$ describes the affinity. The factors $D$, $C$, and $E$ describe the allosteric interactions among the gate, voltage sensors, and Ca$^{2+}$ sensors: the greater the values, the stronger the interactions. When $D = C = E = 1$, there is no allosteric interaction. Because many of our experiments were performed in the virtual absence of intracellular Ca$^{2+}$, the Ca$^{2+}$ sensor-related constants ($K$, $C$, and $E$) shown in blue do not come into play, simplifying the model to the subset shown in black. (B and C) Voltage dependence of open probability ($P_{\text{open}}$) on logarithmic (Left) and linear scale (Right). At $\leq 50$ mV, $P_{\text{open}}$ values were determined from single-channel measurements. At more positive voltages, $P_{\text{open}}$ values were derived from macroscopic recordings. Control data (open circles) were fit with the HA model with the following parameter set (black solid curves): $L_0 = 7.8 \times 10^{-6}$; $z_L = 0.29$; $V_{HA0.5} = 122$ mV; $z_J = 0.69$; $D = 9.2$. $P_{\text{open}}$ data in the presence of Cym04 (gray filled circles) were fit by introducing changes either in $L$ only (B, red curve) or in both $J$ and $L$ (C, green curve). For comparison, best fits to Cym04 data obtained changing $J$ only are shown in blue.

Analysis of channel activation for mutant F315Y. (A) Superposition of Slo1-F315Y current traces for 400-ms depolarizations to the indicated voltages before (black) and after application of 10 $\mu$M Cym04 (red). (B) Voltage dependence of tail currents before (gray) and after Cym04 application (red), with superimposed Boltzmann functions. (C) Comparison of parameters characterizing the voltage dependence for Slo1 and Slo1-F315Y using short pulses of 10 ms (Upper) and using extended pulse protocols (Lower) of increasing lengths (Slo1: 1 ms/10 mV; F315Y: 20 ms/10 mV) with less depolarization, starting with 10-ms depolarizations to 300 mV for Slo1 and 200-ms depolarization to 150 mV for Slo1-F315Y.
Fig. S6. Analysis of the linker with respect to Cym04-induced shifts in $V_{0.5}$. (A) Alignment of the S6/RCK linkers for Slo1 and Slo1_9, as well as an illustration of where deletions of residues were made. (B) Half-maximal activation voltage ($V_{0.5}$) and apparent voltage dependences ($V_e$), as well as their shifts induced by 10 μM Cym04. The extended pulse protocol (Fig. S5) was used.

Fig. S7. NS1619 activates Slo1 BK channels via affecting the voltage sensor equilibrium. (A and B) Open probabilities ($P_{\text{open}}$) were determined before (open symbols) and 2 min after application of 30 μM NS1619 (red symbols) and plotted against voltage on linear (A) and logarithmic scale (B). Control data (open circles) were fit with the HA model with the following parameter set (black solid curves): $L_0 = 3.4 \times 10^{-6}$; $z_L = 0.25$; $V_{HA0.5} = 115$ mV; $z_J = 0.85$; $D = 13.3$. To describe channel activation in the presence of NS1619 (red), $V_{HA0.5}$ was reduced to 80 mV and $L_0$ was increased to $5.8 \times 10^{-6}$. Green boxes in B highlight the attenuated channel activation by NS1619 at −50 compared with 50 mV, which is indicative of changes in the equilibrium constant $J$. 

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Fig. S8. Coexpression of Slo1 and Slo1_9a in HEK 293 cells. (A) Normalized mean current recordings at 200 mV from HEK 293 cells expressing Slo1 (slow kinetics, black), Slo1_9a (fast kinetics, gray), and combinations with 1:7 (blue, Slo1:Slo1_9a) and 7:1 (red, Slo1_9a:Slo1) of respective DNA used for transfection. The dashed curves are predictions assuming an independent contribution of Slo1 and Slo1_9a channels. The number of cells used for averaging is indicated in B. (B) Shifts in $V_{0.5}$ upon application of 10 μM Cym04 for the indicated types of DNA transfected. Numbers in parentheses indicate the number of cells measured. (C) Correlation of half-maximal channel activation voltage ($V_{0.5}$) and its shift by Cym04 ($\Delta V_{0.5}$) for cells measured with varying ratios of Slo1 and Slo1_9a DNA used for transfection.