A pharmacological master key mechanism that unlocks the selectivity filter gate in K⁺ channels

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Potassium (K⁺) channels have been evolutionarily tuned for activation by diverse biological stimuli, and pharmacological activation is thought to target these specific gating mechanisms. Here we report a class of negatively charged activators (NCAs) that bypass the specific mechanisms but act as master keys to open K⁺ channels gated at their selectivity filter (SF), including many two-pore-domain K⁺ (K₂P) channels, voltage-gated hERG (human ether-a-go-go-related) gene) channels and calcium (Ca²⁺)-activated big-conductance potassium (BK) type channels. Functional analysis, x-ray crystallography, and molecular dynamics simulations revealed that the NCAs bind to similar sites below the SF, increase pore and SF K⁺ occupancy, and open the filter gate. These results uncover an unrecognized polypharmacology among K⁺ channel activators and highlight a filter gating machinery that is conserved across different families of K⁺ channels with implications for rational drug design.

**ION CHANNELS**

Pharmacological activation of specific types of K⁺ channels has therapeutic potential for the treatment of a variety of disease states, including epilepsy, arrhythmias, vascular constriction, and various pain conditions (1, 2). Consequently, screening efforts have identified a number of agents that open different types of K⁺ channels (2), presumably by targeting their respective channel-specific activation mechanisms.

Distinct structural mechanisms enable K⁺ channels to respond to a plethora of physiological stimuli, including voltage, temperature, mechanical force, and various second messengers, such as adenosine triphosphate (ATP), Ca²⁺, and H⁺, as well as bioactive lipids such as phosphatidylinositol 4,5-bisphosphate (PIP₂) and arachidonic acid (3, 4). However, despite this complexity, these activation pathways seem to converge on the two principal mechanisms known to gate K⁺ channels open: dilation of the “lower” gate at the intracellular pore entrance by inwardly rectifying (Kir) (5) and voltage-gated (Kv) K⁺ channels (6), and activation of the selectivity filter (SF) gate used by most two-pore-domain K⁺ (K₂P) channels (6, 7, 8) and Ca²⁺-activated big-conductance K⁺ (BKCa) channels (9, 10). In voltage-gated hERG (human ether-a-go-go-related) gene) channels, both mechanisms coexist, with voltage opening the lower gate but rapid inactivation occurring through closure of the SF gate (11, 12). Here we identify a common mechanism for drug-induced channel opening that bypasses these physiological activation mechanisms in SF-gated K⁺ channels.

For the mechanosensitive K₂P channels TREK-1 and TREK-2, the voltage-gated hERG channel, and the Ca²⁺-activated BKCa channel, a series of small-molecule activators all harboring a negatively charged group (tetrazole or carboxylate) have been proposed to act as selective channel openers (13, 14). However, application of these compounds to their respective “nontarget” channels revealed an unexpected polypharmacology: All three openers displayed equal efficiency in opening TREK-1 channels (15) and hERG channels (16, 17), as well as BKCa channels (Fig. 1C), whose activation curve is strongly shifted to more negative voltages (Fig. 1C). This suggests that these activators may not target channel-specific activation mechanisms and may instead share a common mechanism. In all cases, the compound-mediated effect was effectively antagonized by large quaternary ammonium ions (QAm⁺) such as tetra-pentylammonium (TPenA) or tetra-hexylammonium (THexA) that are known to block K⁺ channels at a site immediately below the inner entrance to the SF (16) (Fig. 1, A and B, and Fig. SIC). Likewise, all these activators reduced the QA⁺-mediated inhibition in these different K⁺ channels (Fig. 1C and figs. S1, A and B, and S7, A and B). Furthermore, extended screening with BL-1249 also revealed potent activation of several other K₂P channels (TREK-2, TRAAK, TALK-1, TALK-2, THIK-1, and THIK-2; fig. S1D). Together, these data suggest that these negatively charged activators (NCAs) (BL-1249, PD-118057, and NS11021) act on a gating mechanism that is shared among these different classes of K⁺ channels and that their action involves a site that overlaps with the conserved QA⁺-binding site located below the SF.

A distinctive feature of all NCA-responsive K⁺ channels is their gating by the SF, a mechanism that is intimately coupled to ion permeation (17, 18). In K₂P channels, this coupling leads to pronounced activation by Rb⁺, which displays an ion occupancy distinct to K⁺ at the four SF K⁺ binding sites (S1 to S4) that stabilizes the activated state of the SF gate (17). Interestingly, Rb⁺ not only activated all NCA-responsive K₂P channels but also led to robust activation of BKCa and hERG channels (Fig. 1D). By contrast, Rb⁺ failed to exert any activatory effect on K⁺ channels gated at the helix-bundle crossing (i.e., Kir and most Kv channels), as was observed for Kᵨ.11, Kᵨ.15, Kᵨ.3.1, and Kᵨ.11 (Fig. 1D); consistent with this, these channels were also not activated by BL-1249 (fig. S2, A to E). Furthermore, cyclic nucleotide-gated channels that are also gated at the SF were not activated by BL-1249, indicating that the NCA mechanism may be specific to SF-gated K⁺ channels (fig. S2F).

To gain further mechanistic insight into channel opening by the NCAs, we next investigated their binding by x-ray crystallography, cysteine-scanning mutagenesis, and atomistic molecular dynamics (MD) simulations. First, anomalous diffraction data were collected from TREK-2 channels co-crystallized with a brominated derivative of BL-1249 (BL-1249Br) (Fig. 2A; fig. S3, A to C, and supplementary materials and methods). Although no discrete electron density was visible for BL-1249 itself, in anomalous difference maps, two bromine peaks were clearly visible per TREK-2 dimer (fig. S3, A and B) and the main-chain protein backbone showed excellent agreement with a previously crystallized high-resolution structure of TREK-2 [Protein Data Bank (PDB) 4XDJ (19)]. Both bromine anomalous difference peaks were located at the entrance of the side fenestrations branching off the central pore cavity below the SF. Comparison with a structure
that included QAes− (16) showed that these bromine positions reside within the spherical volume of THexA but outside that of the smaller tetra-ethyl-ammonium (TEA) ion. Consistent with this, BL-1249 activation of TREK-2 channels was antagonized by THexA but not by TEA (Fig. 2, B and C).

These structural data were complemented by cysteine-scanning mutagenesis of the pore-lining M2 and M4 helices of TREK-1. Six residues, including the highly conserved Pro393 (P183) and Leu304 (L304) (also investigated in TREK-2, fig. S3D), were identified where mutations markedly reduced the apparent affinity of BL-1249. These residues cluster around the bromine densities.
detected in the TREK-2 cocrystal with BL-1249Br (Fig. 2D and fig. S3, C and D). A role for L304 in this presumed binding site was further supported by cysteine-modification protection experiments in which the time course of irreversible pore blockade induced by application of the cysteine-modifying agent MTS-TBAO \([8-\text{(tributylammonium)}\text{octyl methanethiosulfonate}]\) (20) to TREK1 L304C (Leu183→Cys) channels was markedly slowed by the presence of BL-1249 (Fig. 2, E and F). This effect was specific for BL-1249, as two further channel activators with distinct binding sites [2-APB at the C terminus (27) and ML335 behind the SF (22)] both failed to slow this rate (Fig. 2, E and F). Furthermore, TREK-1 activation with 2-APB or ML335 was not antagonized by QA+ inhibition, and mutations at the BL-1249 site did not affect 2-APB activation (fig. S4, A, B, and E).

In addition, we performed MD simulations to examine the orientation of BL-1249 within its proposed binding site (Fig. 2G). The favored binding pose oriented the negatively charged tetrazole group of BL-1249 toward the S6 “cavity binding site” for \(K^+\) just below the SF. The remainder of the BL-1249 molecule engaged with residues in M2 and M4 consistent with our scanning mutagenesis data (Fig. 2D and fig. S3D). Moreover, the bromine atom in these simulations was found to be within 3 to 4 Å of the bromine densities determined by crystallography (fig. S3E). Together, these data indicate that BL-1249 binds to a site below the SF and reveal a critical role of the negative charge of the acidic tetrazole ring (pKₐ around 5, where Kₐ is the dissociation constant), implying a pH-dependent compound efficacy. Indeed, when tested with the \(K_{2P}\) channel TALK-2 [exhibiting little intrinsic intracellular pH (pHi) sensitivity], BL-1249 potency dropped strongly with a lowering of the solution pHi to 5, whereas control experiments with 2-APB lacked this pH dependence (Fig. 3F).

We have recently used atomistic MD simulations and a double-bilayer setup to study ion permeation in the TRAAK \(K_{2P}\) channel (47). Therefore, we carried out simulations of ion permeation in...
TREK-2, BKCa, and D). In addition, MD simulations of their TRAAK with BL-1249 modeled into the equivalent site in the TRAAK channel structure (PDB 41f9W) (Fig. 3A). This indicated several changes induced by BL-1249: (i) K⁺ occupancy at the S6 site located adjacent to the negatively charged tetrazole group of BL-1249 increased ~16-fold (Fig. 3, A and B), (ii) K⁺ occupancy of the S1 and S4 sites increased (Fig. 3B), and (iii) the rate of K⁺ permeation increased by 1.6-fold (24 ± 2 ions/μs compared to 15 ± 2 ions/μs without BL-1249; Fig. 3C).

The effect of BL-1249 on ion permeation was further investigated with single-channel recordings of TREK-2 expressed in human embryonic kidney (HEK) 293 cells. Besides an increase in open probability, an increase in the measured single-channel amplitude was also observed in both the inward (from ~29.3 ± 1.5 to ~34.1 ± 1.9 pA at ~100 mV; n = 7) and outward (from 17.7 ± 1.3 to 21.7 ± 1.4 pA at ~100 mV; n = 7) directions in response to BL-1249 (Fig. 3, D and E). This result is consistent with the observed increase in SF ion occupancy at S1 and S4 that is expected to enhance ion permeation via a direct knock-on effect for ions entering the SF from either side (23). A similar increase in unitary conductance was also observed for TREK-1 channels recorded in patches from Xenopus oocytes (Fig. 4, A to C). Notably, increases in single-channel conductance have not been observed upon activation of TREK-1, TREK-2, or TRAAK K⁺ channels by other physiological stimuli (24, 25).

Collectively, these results indicate that BL-1249 increases ion permeation and channel-open probability by influencing K⁺ occupancy at sites below and within the SF. In line with this notion, mutations in the SF that change filter ion occupancy at the S1 and S4 sites (17, 26) and induce the activated “leak mode” in K₁ᵥ channels (17) also render them insensitive to BL-1249 (and various other activators discussed below; fig. S6, A to D).

The negatively charged moiety identified within in BL-1249, PD-118057, and NS1021 is also found in a series of known activators of TREK-1 and TREK-2 K₁ᵥ channels [ML67-33 (27), tetrazole; DCPB (28), carboxylate], hERG channels [PD-307243 (29), carboxylate; NS3623 (30), tetrazole] and BKCa channels [GoSlo-SR-5-6 (31), sulphonate], and its requirement for channel activation has been demonstrated for ML67-33 and GoSlo-SR-5-6 (27, 32). Indeed, these compounds also share all the hallmark features of BL-1249 action, including polypharmacology (i.e., mutual activation of K₁ᵥ, BKCa, and hERG channels [Fig. 4, C and D], sensitivity to QA⁺ (Fig. 4A; fig. S7, A to C; and tables S2 and S3] and mutations that reduce BL-1249 activation in TREK-1 (fig. S4, C and D). In addition, MD simulations of their interaction with structures of the TREK-2, BKCa and hERG channel pores identified similar stable binding poses below the SF with orientation of the negative moiety toward the cavity and a concomitant increase in K⁺ occupancy at cavity and SF ion binding sites (Fig. 4, C and D, and figs. S7D and S8, A to C). Notably, this assumed NCA binding site overlaps with the “promiscuous inhibitor binding site” in the hERG channel, which underlies drug-induced long QT syndrome (22, 32). This site is thought to accommodate many hydrophobic molecules (e.g., terfenadine), and consistent with this, we found that activation by PD-118057 strongly reduced inhibition by terfenadine (Fig. 4B).

The molecular features of the NCA compounds define a common pharmacophore that, besides the negatively charged group, comprises both aromatic and hydrophobic moieties (Fig. 4E). As a control, we tested tetrazole-containing compounds that do not fit this common pharmacophore on TREK-1, BKCa, and hERG channels and found that they were unable to promote channel activation (fig. S9, A to C).

Our results uncover a class of K⁺ channel openers, the NCAs, that act as a universal master key to unlock the SF gate. Mechanistically, these NCAs bind below the SF, where their negative charge promotes K⁺ binding to the pore cavity, and thereby also alter the ion occupancy in the SF in a way that is known to promote activation of the filter gate (17). We hypothesize that, in particular, the increase at the S1 and S4 sites is responsible for activating the SF gate because all NCA-responsive channels are also activated by Rb⁺ permeation, which is thought to increase ion occupancy at these sites, whereas mutations known to reduce S1 and S4 ion occupancy in K₁ᵥ channels abolish NCA activation. Furthermore, a loss of K⁺ binding to the S1 site has been implicated in SF inactivation in K₁ᵥ channels (33), hERG channels (32), and TREK-2 K₁ᵥ channels (34). However, at this time, we cannot exclude the possibility that nonelectrostatic interactions of the NCAs with their respective binding sites also contribute to the stabilization of the active SF state because these sites involve gating-sensitive regions (i.e., the TM4 (8, 19) and S6 segments (6, 34)). In any case, our results support the view that many K₁ᵥ channels, as well as BKCa channels, adopt a low-activity (i.e., inactivated) state of their SF at rest and that the various physiological stimuli induce structural changes that drive the SF into an active (open) state. The NCAs appear to operate by means of bypassing these activation mechanisms to directly stabilize the SF in its active state.
In addition, our findings have important implications for the development of drugs that target K⁺ channels, because they reveal the binding sites and the mechanism of action for many established activators in various K⁺ channels. Our findings have also identified the first activators of either K₂P, hERG, or BKCa channels. Competitive antagonism is seen in the presence of QA⁺⁺ (either THexA or TPenA, which produce ~70 to 80% inhibition of respective basal K⁺ currents). ***P ≤ 0.001; error bars indicate SEM.

The bar chart below represents the fold activation of hERG tail currents at −100 mV with 10 µM of the indicated compounds. Error bars indicate SEM; the B and C superscripts indicate the subunit of the tetramer.

In acute situations such as ischemic stroke or status epilepticus, exploiting the polypharmacology of NCAs to promote simultaneous opening of multiple neuroprotective K⁺ channels (e.g., BKCa, TREK-1, TREK-2, TRAAK, THIK-1, and THIK-2) may even be beneficial.

**REFERENCES AND NOTES**

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Science 363 (6429), 875-880.
DOI: 10.1126/science.aav0569

A key to potassium channel activation

Using drugs to activate potassium channels has the potential to treat conditions like epilepsy, heart arrhythmias, and pain. Schewe et al. report a class of negatively charged activators (NCAs) with a defined pharmacore that use a similar mechanism to activate many types of potassium channels. X-ray crystallography, functional analysis, and molecular dynamics simulations showed that the NCAs bind below the selectivity filter to open the filter gate and activate the channels. Targeting this NCA site might be exploited in rational drug design.

Science, this issue p. 875