**INTRODUCTION**

K\(_{\text{2P}}\) channels regulate nervous, cardiovascular, and immune system functions (1, 2) through the action of their selectivity filter (C-type) gate. C-type gating mechanisms, best characterized in homotetrameric potassium channels, remain controversial and are attributed to selectivity filter pinching, dilation, or subtle structural changes. The extent to which such mechanisms control C-type gating of innately heterodimeric K\(_{\text{2P}}\)s is unknown. Here, combining K\(_{\text{2P}2.1}\) x-ray crystallography in different potassium concentrations, potassium anomalous scattering, molecular dynamics, and electrophysiology, we uncover unprecedented, asymmetric, potassium-dependent conformational changes that underlie K\(_{\text{2P}}\) C-type gating. These asymmetric order-disorder transitions, enabled by the K\(_{\text{2P}}\) heterodimeric architecture, encompass pinching and dilation, disrupt the S1 and S2 ion binding sites, require the uniquely long K\(_{\text{2P}}\) SF2-M4 loop and conserved “M3 glutamate network,” and are suppressed by the K\(_{\text{2P}}\) C-type gate activator ML335. These findings demonstrate that two distinct C-type gating mechanisms can operate in one channel and underscore the SF2-M4 loop as a target for K\(_{\text{2P}}\) channel modulator development.

**RESULTS**

Potassium-dependent selectivity filter structural changes

Despite the fact that C-type gating is the principal K\(_{\text{2P}}\) gating mechanism (3–6) and that previously determined K\(_{\text{2P}}\) structures show major conformational changes that affect function (20–26), all prior
K$_{2P}$ structures show identical, canonical selectivity filter conformations and lack changes that could be attributed to C-type gating (fig. S1). Notably, these structures were all determined in the presence of 150 to 200 mM permeant ions, a condition that would be expected to confer considerable C-type gate stabilization based on functional studies (3, 4, 6, 10). In notable contrast, structure determination of a crystallizable K$_{2P}$2.1 (TREK-1) construct, K$_{2P}$2.1$_{TREK}$ (20), under a series of seven potassium concentrations, 0, 1, 10, 30, 50, 100, and 200 mM [K$^+$] at resolutions of 3.9, 3.4, 3.5, 3.3, 3.6, 3.9, and 3.7 Å, respectively, revealed obvious potassium-dependent changes in the selectivity filter structure, particularly in SF2 and the SF2-M4 loop (Fig. 1A, figs. S2 and S3, and table S1). These changes manifested at potassium concentrations ≤50 mM and eventually encompassed all of the SF2-M4 loop and the upper portion of the selectivity filter (Gly$^{253}$-Lys$^{271}$) (figs. S2 and S3). Additional changes were observed in SF1 residues Gly$^{144}$, Asn$^{147}$ at the lowest potassium concentrations (0 and 1 mM) (Fig. 1B and fig. S3, A and B). Structure determination under the same set of potassium concentrations in the presence of the K$_{2P}$2.1 (TREK-1) activator ML335 (20) at resolutions of 3.4, 2.6, 3.0, 3.2, 3.2, 3.3, and 3.8 Å, respectively, yielded essentially identical structures having canonical selectivity filter conformations at all potassium concentrations (Fig. 1, C and D, and figs. S2 and S3, A and B), a result that agrees with the ability of ML335 to activate the C-type gate directly (20). The observed structural changes were limited to the SF1 and SF2-M4 regions and were uncorrelated with differences in resolution (fig. S2A). Moreover, other parts of the channel remained well defined even when the SF2-M4 loop became disordered (fig. S2, B and C) and had essentially the same conformations as prior K$_{2P}$2.1 (TREK-1) structures that show the absence of an inner gate (20). Hence, the changes we observe clearly represent a local, specific, potassium-dependent loss of structure.

Structural studies of homotetrameric potassium channels have established the intimate connection between the presence of potassium ions in the selectivity filter and the conductive conformation in which the selectivity filter backbone carbonyls coordinate the permeant ions (11, 13–17). Hence, we asked whether the SF1, SF2, and the SF2-M4 loop structural changes in different potassium concentrations were also accompanied by changes to the number of ions in the filter. Comparison of selectivity filter region omit maps (28) showed clear evidence for variation in the number of ions in the filter that paralleled the structural changes in the filter and supporting loops. The 100 and 200 mM [K$^+$] structures showed ions at all four selectivity filter sites, S1 to S4, similar to prior structures determined under similar conditions (20). Whereas in the 0, 1, 10, 30, and 50 mM [K$^+$] structures, the ion densities at sites S1 and S2 were clearly absent, while the S3 and S4 ions persisted to the lowest potassium concentration examined (Fig. 2A and fig. S4A). By contrast, all of the K$_{2P}$2.1 (TREK-1):ML335 structures showed ions at S1 to S4 regardless of the potassium concentration, underscoring the ability of ML335 to stabilize the filter (Fig. 2B and fig. S4B) and directly activate the C-type gate (20).

To confirm that the changes in the electron density reflected potassium ion occupancy and were not due to resolution differences, we used long-wavelength x-rays above and below the potassium K-absorption edge (λ$_{\text{K}}$ = 3.3509 and 3.4730 Å) to measure potassium anomalous scattering (29, 30) from crystals in 1 or 200 mM [K$^+$] in the absence or presence of ML335. Anomalous difference maps showed unequivocally that potassium ions occupy sites S1 to S4 under 200 mM [K$^+$] conditions irrespective of the presence of ML335 (Fig. 2, C and D). By contrast, the density from 1 mM [K$^+$] conditions showed a ML335-dependent difference in the number of potassium ions (Fig. 2, C and D) that agreed with our initial observations (Fig. 2, A and B, and table S2). In the absence of the activator, potassium ions were observed only in the lower portion of the filter, whereas potassium ions are found at all four positions in presence of ML335 (Fig. 2, C and D). Together, these data demonstrate that the loss of structure observed in the upper portion of SF2 as potassium concentrations are lowered is accompanied by a loss of potassium ions at sites S1 and S2 (Fig. 2E). Hence, the well-ordered, fully ion-bound conformations represent the active state of the filter, whereas the low [K$^+$] structures in the absence of ML335 that have various degrees of disorder in SF1, SF2, and the SF2-M4 loop and lack of ions at S1 and S2 reflect low activity conformations of the C-type gate. This assignment agrees with the idea that K$_{2P}$ C-type gate activation involves a rigidification of the filter and surrounding structure (20).

**C-type gate and connecting loops are dynamic**

To gain further insight into how potassium occupancy and ML335 affect the C-type gate, particularly in the context of a lipid bilayer, we turned to molecular dynamics (MD) simulations of K$_{2P}$2.1
(TREK-1). Initially, we simulated two conditions: (i) 180 mM [K+] and a +40-mV applied membrane potential (denoted “High [K+]/+40 mV,” 36.5 μs aggregate) and (ii) the same [K+] and potential with bound ML335 (denoted “High [K+]/+40 mV/ML335,” 31.6 μs aggregate). Both conditions showed many permeation events (144 and 253 for High [K+]/+40 mV and High [K+]/+40 mV/ML335, respectively), confirming that the initial structures represent conduction competent states. Nevertheless, the pattern of permeation events with respect to time showed notable differences depending on ML335 (Fig. 3A). Over the course of the simulations, most of the High [K+]/+40 mV/ML335 trajectories (8 of 10) remained in a stable, ion-conducting state. By contrast, most (7 of 12) of the trajectories originated from High [K+]/+40 mV/ML335 determined in 0 mM [K+] [pale yellow (4°), blue white (4°)], 50 mM K+ [bright orange (5°); marine (4°)], 100 mM [K+] [olive (4°); slate (4°)], and 200 mM K+ [orange (4°); deep blue (4°)]. Potassium ions are magenta spheres. Sites S1 to S4 are labeled. ML335 is shown as sticks. (A and B) Polder omit maps (S28) for structures of (A) K2P2.1(TREK-1) and (B) K2P2.1(TREK-1):ML335 determined in 0 mM [K+] [pale yellow (4°); blue white (4°)], 50 mM K+ [bright orange (5°); marine (4°)], 100 mM [K+] [olive (4°); slate (4°)], and 200 mM K+ [orange (4°); deep blue (4°)]. Potassium ions are magenta spheres. Sites S1 to S4 are labeled. ML335 is shown as sticks. (C and D) Potassium anomalous difference maps (S29) for (C) K2P2.1(TREK-1) and (D) K2P2.1(TREK-1):ML335 determined in 0 mM [K+] [pale yellow (4°); marine (4°)], and 200 mM K+ [orange (4°); deep blue (4°)]. In (A) and (D), SF1 in the 200 mM K+ conformation is shown for reference. S1 to S4 sites and select amino acids are labeled. (E) Plot of the number of observed selectivity filter ions as a function of [K+]. Colors correspond to the scheme in Fig. 1 (C and D).

To determine whether there were differences in C-type gate dynamics across simulation conditions, we examined a number of factors. Because structural waters behind the selectivity filter stabilize both the active and C-type inactivated states of the model homotrimeric channel KcsA (31), we first characterized the role that water molecules have on the K2P2.1 (TREK-1) filter conformation. We found that in conductive states, regardless of the presence of ML335, a characteristic water network behind the filter stabilizes SF1 and SF2 through hydrogen bonds to the backbone amidates of Phe145/Gly146 and Phe254/Gly255, respectively (fig. S5, A and B). As the K2P2.1 (TREK-1) filter moves away from the canonical, conductive conformation, these well-organized networks dissolve (fig. SSC). Nevertheless, before dissolution, there were no obvious differences in the water configurations with or without ML335 that would explain the differences in conduction and filter stability. We also note that unlike in KcsA, where water molecules stabilize a discrete nonconducting pinched filter state (11, 14, 31), these K2P2.1 (TREK-1)
nonconductive states were heterogeneous, having many different conformations of the filter and surrounding waters.

We next asked whether dynamics in the filter region could explain differences in filter stability. To do so, we calculated root-mean-square fluctuation (RMSF) values for the selectivity filter and the postfilter loops. Because the crystal structures showed that low potassium occupancy in the filter resulted in increased mobility in these regions (Figs. 1, B and D, and figs. S2A and S3), we included a third set of simulations in which K$_{2p}$2.1 (TREK-1) had only a single ion in the filter under no applied membrane potential (denoted “Low [K$^+$]/0 mV,” 20.6 μs aggregate). This analysis revealed that residues Phe$_{145}$–Ser$_{149}$ of SF1, Phe$_{254}$–Gly$_{261}$ of SF2, and the SF2–M4 loop comprise the three most dynamic areas near the filter and showed that their mobility was greatly restricted by ML335 (Fig. 3, C and D). Further, under Low [K$^+$]/0 mV conditions, the mobility of these regions exceeded either of the High [K$^+$]/+40 mV conditions (fig. S5D). Together, the simulations indicate that the absence of K$^+$ in the filter versus the presence of ML335 have strong, opposite effects on the dynamics of the selectivity filter and SF2–M4 loop (Fig. 3, C and D).

To determine specific structural features associated with loss of conductance and how these features relate to the broader C-type gating context, we analyzed the backbone dihedral angles of the SF1 and SF2 ion-coordinating “TIGFG” amino acid motifs. We used a simple statistical procedure known as principal components analysis (PCA) to transform the 10 backbone dihedral angles from each TIGFG conformation into a new coordinate system wherein the greatest variance in conformations lies along the first axis (principle component), the second greatest along the second axis, and so on (32). Focus on the first few high-variance components provides a natural way of reducing the dimensionality of the data and reveals collective changes that cannot be gleaned from examining changes in individual dihedral angles. Projecting all simulation snapshots onto the first two principal components (PC1 and PC2) (Fig. 3E) uncovered a distinct grouping of SF1 and SF2 conformations that lack major deviations from the initial structure. All prior K$_{2p}$ selectivity filter structures (Fig. S6, A and B) (denoted as the “native state”) and selectivity filters from other potassium channels thought to capture either conducting states (14, 33) or, unexpectedly, C-type inactivated states (14, 17), map to the center of this group (fig. S6C). Additional clustering analysis of all High [K$^+$] selectivity filters in simulations under different conditions shows that these structural components display increased conformational disorder and pseudo-fourfold symmetry breaking that is in excellent agreement with the crystallographic ion positions observed under low potassium conditions (Figs. 1 to 3F and figs. S2 and S3). Furthermore, examination of the ensemble of final SF1 and SF2 backbone conformations from the simulations under different conditions shows that these structural components display increased conformational disorder and pseudo-fourfold symmetry breaking that is in excellent agreement with the x-ray structures (Fig. 3F). SF1 adopts nonnative conformations, particularly around Asn$_{147}$, which pinch the conduction pathway, whereas SF2 preferentially dilates out of the pathway (movies S1 and S2). This asymmetry extends beyond the parts of the filter that directly contact the permeant ions. Although the SF1–M2 loop remains largely native-like, despite the changes in SF1, the longer SF2–M4 loop is highly mobile (movie S2). This later observation agrees well with the loss of density for SF2–M4 loop in the low [K$^+$] crystal structures (Fig. 1, A and B). Together, the structures and simulations support the idea that ML335 acts by stabilizing the K$_{2p}$ selectivity filter in a conductive state and indicate that the low [K$^+$] crystal structures represent an inactive C-type gate in which asymmetric disorder in the extracellular portion of the selectivity filter disrupts the S1 and S2 ion binding sites and inhibits ion conductance.

**ML335 stabilizes the K$_{2p}$2.1 (TREK-1) open state**

Both the crystallographic and computational data strongly suggest that ML335 stabilizes the conductive state of the C-type gate. To test this idea directly, we recorded K$_{2p}$2.1 (TREK-1) single channels alone and in the presence of ML335 (Fig. 4, A and B). ML335 activated the channels in the same way regardless of whether it was applied to the bath (Fig. 4A) or through the pipette (Fig. 4B). The effect of bath application was apparent in ~15 min, whereas the pipette application had immediate effects in line with the fact that the K$_{2p}$ modulator pocket faces the extracellular solution. The data clearly show that in both cases, ML335 increases channel open probability but not the single-channel conductance (Fig. 4, C to E). By contrast, the activator BL-1249, which is thought to act by a mechanism different from that of the K$_{2p}$ modulator pocket activators ML335 and ML402 (37, 38), increases both open probability and single-channel conductance (38). The clear effects of ML335 on channel open probability
match the expectations from the crystallographic and computational observations that show that ML335 stabilizes the ion-filled conductive state of the selectivity filter C-type gate (Figs. 1 to 3) and support the idea that rigidification of the P1-M4 interface, comprising the K_2P modulator pocket, is central to C-type gate activation of K_2P8 (20).

**The SF2-M4 loop integrates responses from diverse gating cues**

In most potassium channels, including the first K_2P pore domain (PD1), a six-residue loop connects the extracellular end of the selectivity filter to the outer transmembrane helix of the pore domain (Fig. 5, A and B, and fig. S7A, and B). K_2P8 are unique in that the second pore domain loop (PD2) is longer than this canonical length by six to eight residues in 14 of the 15 K_2P subtypes (fig. S7, C and D). Despite these differences, the N-terminal portions of the PD1 and PD2 loops adopt very similar structures up to Pro150-Gly259, respectively (Fig. 5A). The simulations revealed that loss of SF2-M4 loop stability was accompanied by the disruption of a hydrogen bonding network, the Glu234 network, at the C-terminal end of the selectivity filter (Figs. 1 to 3, and figs. S7, E and F), and loop dynamics are enhanced (Fig. 3D). Together, these results suggest that loop dynamics are important for C-type gating, with Glu234 playing a key role by supporting the SF2-M4 loop structure. Notably, the equivalent position of the K_2P PD1 outer helix, M1 also has a highly conserved glutamate (Fig. S7G) that affects C-type gating through interactions with the short SF1-M2 loop (10, 39) in a manner that is conserved with voltage-gated potassium channels (40, 41). Therefore, the given indications from our structures and simulations that Glu234 network integrity should be important for gating, we set out to test consequences of restricting the SF2-M4 loop mobility and disrupting the Glu234 network.

To create a channel having symmetric length loops between each selectivity filter and its outer transmembrane helix, we transplanted Pro150-Gly259 from PD1 onto PD2, denoted "Loop2syn-6" (Fig. 5B). Loop2syn-6 showed blunted responses to temperature (Fig. 5, E and F) and pressure (Fig. 5, G and H). Consistent with the deletion of key ML335-binding SF2-M4 loop residues, Loop2syn-6 was unresponsive to ML335 (Fig. 5, I and J) but remained partially sensitive to BL-1249 (Fig. 5, K and L), an activator that affects the channel from a site under the selectivity filter (37, 38). Measurement of rectification in inside-out patches, a parameter that is a direct measure of C-type gate activation (5, 20), demonstrated that unlike gain-of-function mutants (20), Loop2syn-6 does not have a constitutively activated C-type gate that would render it insensitive to gating commands (fig. S8, A and B). Hence, the blunted responses caused by shortening the SF2-M4 loop to the canonical length indicate that the unusual length of the SF2-M4 loop is central to C-type gate control.

Disruption of the Glu234 hydrogen bond network by E234Q and Y270F mutations resulted in channels having severely blunted responses to temperature (Fig. 5, E and F), pressure (Fig. 5, G and H), ML335 (Fig. 5, I and J), and BL-1249 (Fig. 5, K and L). Unlike Loop2syn-6, both mutations compromised ion selectivity as evidenced by an altered reversal potential (Fig. 5, E, G, I, and K, and fig. S9). This baseline selectivity defect was partially corrected by temperature or pressure activation (Fig. 5, E and G, and fig. S9). Inside-out patch clamp experiments demonstrated that neither mutant resulted in channels having a C-type gate that was activated...
at rest, although Y270F caused a slight decrease of the rectification coefficient (fig. S8, A and B). Unexpectedly, we also found that E234Q exhibited a time- and voltage-dependent inactivation (fig. S8, C and D), further validating the importance of the Glu234 network for C-type gate control. Together, with prior mutational studies suggesting a role for the SF2-M4 loop in external pH gating (42), these data strongly support the key role that the SF2-M4 loop has in K2P channel gating and underline the importance of SF2-M4 stabilization by the network centered on Glu234.

The M3 glutamate network has a conserved role in C-type gate control

The key elements of the Glu234 network are highly conserved among K2PS (fig. 5D). To test its general importance, we disrupted this
network in $K_{2P}$3.1(TASK-1), a $K_{2P}$ from a subfamily distant from $K_{2P}$2.1 (TREK-1) (2). Structural comparison shows that $K_{2P}$3.1(TASK-1) Glu$^{182}$, Leu$^{208}$, and Tyr$^{220}$ form a network similar to the $K_{2P}$2.1 (TREK-1) Glu$^{234}$-Gly$^{260}$-Tyr$^{270}$ network (fig. S10A). Notably, this network is structurally conserved although $K_{2P}$3.1(TASK-1) has one of the longest SF4-M4 loops (14 residues) (Figs. 5D and fig. S7D) and has a large sidechain, leucine, at the position that contributes the backbone amide (figs. 5, C and D). Disruption of this network in $K_{2P}$3.1(TASK-1) had substantial functional consequences. $K_{2P}$3.1(TASK-1) E182Q failed to produce functional channels (fig. S10C), whereas $K_{2P}$3.1(TASK-1) Y220F yielded channels that were more readily closed by low pH (fig. S10, D and E). This result phenocopies disruption of interactions on the opposite side of the SF2-M4 loop in P1-M4 interface by the $K_{2P}$3.1(TASK-1) I88G mutant (3) and indicates that the Y220F mutation destabilized the SF2-M4 loop and C-type gate. Together, our data demonstrate that the Glu$^{234}$ network and its stabilization of the SF2-M4 loop is a central element of C-type gate control. Because of its conservation and functional importance in diverse $K_{2P}$s, we term this network as the M3 glutamate network.

**DISCUSSION**

**Mechanistic implications for $K_{2P}$ channel function**

Despite the central role of the selectivity filter C-type gate in $K_{2P}$ channel function (3–6), observation of conformational changes that would provide a framework for understanding the principles of $K_{2P}$ C-type gating has eluded previous structural studies (20–26, 43). Our data establish that control of the $K_{2P}$ C-type gate involves unprecedented, asymmetric, potassium-dependent, order-disorder transitions in the selectivity filter and surrounding loops (Figs. 1 and 6). The selectivity filter conformational changes associated with $K_{2P}$ C-type gating comprise two classes of rearrangements that eliminate the S1 and S2 ion binding sites (Fig. 6 and movies S3 and S4). One pinches the SF1 extracellular side and exposes the Asp$^{147}$ side-chains to the extracellular solution (Fig. 6A and movie S3), a position that modulates C-type inactivation in homotetrameric potassium channels (44, 45) and that undergoes similar changes in human Ether-a-go-go-Related potassium channel (hERG) simulations (46). Hence, this class of C-type gating mechanism is shared with other potassium channels. The second unwinds SF2 and the SF2-M4 loop, dilates the selectivity filter along the SF2 axis (Fig. 6B and movie S4), depends on the structure of the uniquely long $K_{2P}$ SF2-M4 loop, and is unlike any of the prior structural changes associated with C-type gating (20–26, 43). SF1 pinching and SF2 dilation are not mutually exclusive and are likely to be interdependent given the role of the SF ions in stabilizing the filter. Such asymmetric changes could contribute to the bimodal distribution of closed state dwell times reported for $K_{2P}$2.1-(TREK-1) (47) and the closely-related $K_{2P}$10.1 (TREK-2) (48). Further, as $K_{2P}$ heterodimer formation yields channels having two unique SF1-M2 and SF2-M4 loops, this structural diversification together with the two nonmutually exclusive inactivation modes likely provides a mechanism for the emergence of heterodimer properties that differ from either homodimer parent (49–56). The structural rearrangements in the pore and surrounding regions, loss of S1 and S2 ions, and the demonstration that destabilization of the SF2-M4 loop structure compromises ion selectivity are reminiscent of studies of the nonselective bacterial channel NaK, which has only the S3 and S4 sites and can be converted into a potassium-selective channel by forming the S1 and S2 ion binding sites (27). Further, the loss of ion selectivity associated with $K_{2P}$ C-type gating (4, 10, 57) and the strong link between $K_{2P}$ gating and external potassium concentration (3, 4, 6, 10) are in good accord with the structural and functional changes we observe.

Although C-type gating is an important mode of channel regulation in many potassium channel classes (38), structural insights into its mechanistic basis are limited to studies of a small number of homotetrameric potassium channel types (11, 13–19) and lack consensus (12), even for the best studied example, KcsA (58–61). Nevertheless, our studies identify a unifying feature shared between $K_{2P}$ C-type gating and homotetrameric potassium channel C-type gating—the importance of the conserved glutamate at the extracellular end of the pore module outer helix (Figs. 5, C and D, and fig. S7G). This site on the $K_{2P}$ PDI M1 helix affects C-type gating through interactions with the SF1-M2 loop (10, 39) similar to other channels having a canonical six residue loop between the selectivity filter and pore module outer helix (Fig. 5B) (40, 41, 62). The equivalent PD2 glutamate on $K_{2P}$21 (TREK-1) M3, Glu$^{234}$, forms a conserved network together with a M4 tyrosine, Tyr$^{270}$, the M3 glutamate network that supports the uniquely long SF2-M4 loop found throughout the $K_{2P}$ family (fig. S7D). Disruption of the M3 glutamate network blunts responses to diverse stimuli in distantly related $K_{2P}$s (Fig. 5 and figs. S9 and S10) and establishes that, together with its role in external pH responses (42), the SF2-M4 loop is a hub that integrates chemical and physical gating cues sensed in other parts of the channel (Fig. 5, E to L) and relayed to the filter via M4 (3, 4). The M3 glutamate network is conserved in every functional $K_{2P}$ except $K_{2P}$18.1 (TRESK), the only $K_{2P}$ having a short SF2-M4 loop (Fig. 5D and fig. S7D). This conservation, together with the report that a pulmonary hypertension mutation at the conserved M3 glutamate in $K_{2P}$3.1 (TASK-1), E182K, disrupts function (63) underscores the importance of the M3 glutamate network and SF2-M4 loop in gating throughout the $K_{2P}$ family.

Our studies establish that $K_{2P}$ channel C-type gating entails filter pinching (SF1) and pore dilation (SF2), highlight the dynamic nature of C-type inactivated states (20, 64), and indicate that the innate heterodimeric nature of the $K_{2P}$ filter architecture enables two general C-type gating mechanisms, pinching and dilation (12), which have been viewed as mutually exclusive, to operate in one channel. The substantial differences in the degree of conformational changes between SF1 and SF2 appear to depend on the loop length connecting these elements to the outer transmembrane helix of their respective pore domains. Binding of small molecules, such as ML335, to the $K_{2P}$ modulator pocket enables conduction by stabilizing the SF2-M4
loop and selectivity filter and increasing channel open probability, whereas disruption of the integrity of the SF2-M4 loop blunts transduction of gating cues that originate from the intracellular C-terminal tail (3, 65–70) and pass through M4 to the C-type gate (3, 4). These findings corroborate the idea that the K_{2,1} selectivity filter and its supporting architecture are dynamic under basal conditions (20), that ion permeation requires limiting filter mobility through ligand binding to the K_{2,1} modulator pocket or by conformational changes transmitted through the M4 helix (20), that permeant ions organize and stabilize the K_{2,1} conductive state (5, 38), and that the inactive state involves an ion-depleted filter (5). Further, our observation that the filter can adopt nonconductive conformations although the M4 transmembrane helix is in the “up” position underscores previous studies indicating that M4 conformation is not the sole determinant of K_{2,1} activation (20, 71). The key role for the SF2-M4 loop in transducing gating cues sensed by intracellular channel components to the K_{2,1} selectivity filter gate such as temperature and pressure (Fig. 5, E to H), as well as external pH responses (42), demonstrates its pivotal function in K_{2,1} gating. These properties, together with the ability of ML335 to increase open probability by stabilizing this loop (Figs. 1 to 5), explain why the P1-M4 interface, which is framed on one side by the SF2-M4 loop, is central to K_{2,1} gating (3, 4, 20) and why small molecules bound to this interface activate the channel (20). These findings emphasize the potential for targeting this unique K_{2,1} loop for selective small molecule or biologic modulators directed at K_{2,1}-dependent processes such as anesthetic responses (72, 73), pain (74–76), arrhythmia (77), ischemia (72, 78), and migraine (52).

**MATERIALS AND METHODS**

**Protein expression and purification**

An engineered mouse K_{2,1} (TREK-1), denoted K_{2,1}cryst, encompassing residues 21 to 322 and bearing the following mutations: K84R, Q85E, T86K, I88L, A89R, Q90A, A92P, N95S, S96D, T97Q, N119A, S300A, E306A, a C-terminal green fluorescent protein (GFP), and His_{10} tag was expressed and purified from *Pichia pastoris* as previously described (20).

**Crystallization and refinement**

Purified K_{2,1}cryst was concentrated to 6 mg ml⁻¹ by centrifugation (Amicon Ultra-15, 50 kDa molecular mass cutoff; Millipore) and crystallized by hanging-drop vapor diffusion at 4°C using a mixture of 0.2 μl of protein and 0.1 μl of precipitant over 100 μl of reservoir containing 20 to 25% polyethylene glycol 400 (PEG400), 200 mM KCl, 1 mM CdCl₂, and 100 mM Hepes (pH 8.0). Crystals appeared in 12 hours and grew to full size (200 to 300 μM) in about 1 week.

Crystals were harvested and cryoprotected with buffer D [200 mM KCl, 0.2% octyl glucose neopentyl glycol (OGNG), 15 mM n-heptyl-β-D-thiogluicoside (HTG), 0.02% cholestery hemisuccinate (CHS), 1 mM CdCl₂, and 100 mM Hepes (pH 8.0)] with 5% step increases of PEG400 up to a final concentration of 38%. After cryoprotection, crystals were incubated for 8 hours in buffer E [38% PEG400, 0.2% OGNG, 15 mM HTG, 0.02% CHS, 1 mM CdCl₂, and 100 mM Hepes (pH 8.0)] containing 200 mM salt consisting of NaCl and KCl in varied proportions to yield the following K⁺ concentrations: 0, 1, 10, 30, 50, 100, and 200 mM. In the soaking experiments where the activator was present, ML335 was added to the soaking cocktail to a 1 mM final concentration. The nominal K⁺ concentration in the 0 mM condition is ~20 nM. Crystals were subsequently harvested and flash-frozen in liquid nitrogen.

Datasets for K_{2,1}cryst in the presence of differing potassium concentrations, alone or with ML335, were collected at 100 K using synchrotron radiation at advanced photon source (APS) GM/CAT beamline 23-IDB/D Chicago, Illinois, processed with XDS (79), scaled, and merged with Aimless (80). Final resolution cutoffs were 3.9, 3.5, 3.4, 3.3, 3.6, 3.9, and 3.7 Å for K_{2,1}cryst in the presence of 0, 1, 10, 30, 50, 100, and 200 mM potassium, respectively, and were arrived at using the CC₁/₂ criterion and standard best practices based on map quality (81). Final resolution cutoffs for the K_{2,1}cryst:ML335 complexes were 3.4, 2.6, 3.0, 3.2, 3.2, 3.3, and 3.8 Å in the presence of 0, 1, 10, 30, 50, 100, and 200 mM potassium, respectively. Structures were solved by molecular replacement using the K_{2,1}cryst structure [Protein Data Bank (PDB): 6CQ6] (20) as search model purged of all the ligands. The best resolution structure (1 mM:ML335) had density for head group of the lipid in the phosphatidylinositol 4,5-bisphosphate (PIP₂) binding site and was built accordingly. Several cycles of manual rebuilding, using COOT (82), and refinement using REFMAC5 (83) and PHENIX (84) were carried out to improve the electron density map. Twofold local automatic noncrystallographic symmetry restraints were used during refinement.

Two potassium ions were modeled into 2Fo-Fc densities of the Apo K_{2,1}cryst 0, 1, 10, and 50 mM structures; whereas, four potassium ions were modeled into 2Fo-Fc densities of the Apo K_{2,1}cryst 100 and 200 mM structures. Four potassium ions were modeled for all the K_{2,1}cryst:ML335 complexes. To validate the presence of the potassium ions, a polder map (28) was generated for each structure. The polder map of the Apo K_{2,1}cryst 50 mM structure showed a density in the filter that extended beyond the S3 site into the S2 site; however, modeling an additional low occupancy K⁺ ion at this site did not improve the overall statistics. Attempts to refine the occupancy of this third ion using PHENIX (84) yielded an ion having zero occupancy. Hence, the final structure has two ions in the filter, although there may be a low occupancy ion present that is not accountable due to the resolution limit of the data. The final cycle of refinement of each structure was carried out using BUSTER (85).

**K⁺ anomalous data collection**

Long-wavelength data were collected at beamline I23, Diamond Light Source (30), UK, at a temperature ~ 50 K at wavelengths of 3.3509 and 3.4730 Å, above and below the potassium K absorption edge, processed and scaled with XDS/XSCALE (79). Anomalous difference Fourier maps to locate the potassium positions were calculated with ANODE (86) using the K_{2,1} (TREK-1) structure (PDB:6CQ6) (20). Peaks present in the maps above but absent in the maps below the absorption edge were assigned as potassium.

**Two-electrode voltage-clamp electrophysiology**

Two-electrode voltage-clamp recordings were performed on defolliculated stage V to VI *Xenopus laevis* oocytes 18 to 48 hours after microinjection with 1 to 40 ng of mRNA. Oocytes were impaled with borosilicate recording microelectrodes (0.3- to 3.0-MΩ resistance) backfilled with 3 M KCl. Except where otherwise indicated, recording solution was 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, and 1.0 mM MgCl₂ buffered with 5 mM Hepes at pH 7.4 and was perfused by gravity. For pH_{4} experiments, the standard buffer was replaced with 10 mM tris (pH 9.0 and 8.1), 5 mM Hepes (pH 7.8 and 7.1), or 5 mM MES (pH 6.5 and 5.9).
Currents were evoked from a −80-mV holding potential followed by a 300-ms ramp from −150 to +50 mV. Data were acquired using a GeneClamp 500B amplifier (MDS Analytical Technologies) controlled by pCLAMP software (Molecular Devices) and digitized at 1 kHz using Digidata 1332A digitizer (MDS Analytical Technologies).

For temperature experiments, recording solutions were heated by an SC-20 in-line heater/cooler combined with an LCS-1 liquid cooling system operated by the CL-100 bipolar temperature controller (Warner Instruments). Temperature was monitored using a CL-100–controlled thermistor placed in the bath solution 1 mm upstream of the oocyte. For temperature experiments, perfusate was warmed from 15°C to 35°C in 5°C increments, with recordings performed once temperature readings stabilized at the desired values. Temperature response data were fit with the equation $A = A_{\text{min}} + (A_{\text{max}} - A_{\text{min}}) / (1 + e^{(T - T_0)/\Delta T})$ where $A_{\text{min}}$ and $A_{\text{max}}$ are the temperature and maximum activation, respectively, $T_0$ is the temperature of half maximal activation, and $\Delta T$ is the slope factor ($\Delta Q$).

For pH experiments, solutions were exchanged consecutively from 9.0 to 5.9 while maintaining the temperature at 22.5°C. pH response data were fit with the equation $A = A_{\text{min}} + (A_{\text{max}} - A_{\text{min}}) / (1 + ([H+]_o/K_{\text{H}})^{1/2})$ where $A_{\text{min}}$ and $A_{\text{max}}$ are the minimum and maximum activation, respectively, $K_{\text{H}}$ is the Hill slope.

Dose-response experiments were conducted at room temperature (22°C) and used standard recording solution at pH 7.4 supplemented with 0.2% dimethyl sulfoxide and the indicated concentration of ML335 (20). Dose-response data were fit with the equation $A = A_{\text{min}} + (A_{\text{max}} - A_{\text{min}}) / (1 + (EC_{50}/[ML335])^{1/2})$ where $A_{\text{min}}$ and $A_{\text{max}}$ are the minimum and maximum activation, respectively, $EC_{50}$ is the half maximal effective concentration, and $H$ is the Hill slope. Data analysis and curve fitting were performed using Clampfit and Python according to procedures adapted from (4, 20). X. laevis oocytes were harvested from female X. laevis according to UCSF Institutional Animal Care and Use Committee (IACUC) Protocol AN178461.

**Patch clamp electrophysiology**

Human embryonic kidney cells (HEK293) were grown at 37°C under 5% CO2 in a Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 10% l-glutamine, and antibiotics (penicillin (100 IU ml−1) and streptomycin (100 mg ml−1)). Cells were transfected (in 35-mm-diameter wells) using Lipofectamine 2000 (Invitrogen) and a pIRE-GFP (Invitrogen) plasmid vector into which the gene encoding for mouse K2P2.1 (TREK-1) wild type or mutants has been inserted in the first cassette (4). DNA (1 μg) was used for K2P2.1 (TREK-1) and Loop2-sym-6, whereas 3 μg of DNA was necessary to record reliable currents from E234Q and Y270F. Data acquisition was performed using pCLAMP 10 (Molecular Devices) and an Axopatch 200B amplifier (Molecular Devices).

The inside-out configuration of the patch clamp technique was used to record K+ or Rb+ currents at room temperature (23°C ± 2°C) 24 to 48 hours after transfection (5, 20). Pipettes were pulled from borosilicate glass capillaries (TW150F-3, World Precision Instruments) and polished (MF-900 microforge, Narishige) to obtain 1- to 2-MΩ resistances.

Stretch activation of K2P2.1 (TREK-1) and mutants was performed by applying a −50-mmHg pressure to the inside-out patch through a high-speed pressure clamp (HSPC-1, ALA Scientific Instruments) connected to the electrode suction port, after recording the current at 0 mmHg. Pipette solution contained 150 mM NaCl, 5 mM KCl, 1 mM CaCl2, 2 mM MgCl2, and 20 mM Hepes (pH 7.4 with NaOH). Bath solution contained 145 mM KCl, 3 mM MgCl2, 5 mM EGTA, and 20 mM Hepes (pH 7.2 with KOH) and was continuously perfused at 200 ml hour−1 during the experiment. K2P2.1 (TREK-1) currents were elicited by a 1-s ramp from −140 to +50 mV from a −80-mV holding potential.

Single-channel activity was recorded under the cell-attached configuration of the patch clamp technique, using patch pipettes of about 8 MΩ pulled from quartz glass capillaries (QP100-70-7.5, Sutter Instrument, Novato, CA, USA) in a laser-based micropipette puller (P-2000, Sutter Instrument). Both the pipette and bath solutions contained 150 mM KCl, 5 mM EGTA-K, 1 mM EDTA-K, and 10 mM Hepes (pH 7.3 with KOH). Currents were low-pass–filtered at 2 kHz and digitized at a sampling rate of 20 kHz. Threshold detection of channel openings was set at 50%. Channel activity (NPo, where N is the number of channels in the patch and Po is the probability of a channel being open) was determined from ≥30 s of current recordings.

Voltage-dependent activation and inactivation of K2P2.1 (TREK-1) and mutants were recorded from inside-out patches. Pipette solution contained 150 mM KCl, 3.6 mM CaCl2, and 10 mM Hepes (pH 7.4 with KOH). Bath solution contained 150 mM RbCl, 2 mM EGTA, and 10 mM Hepes (pH 7.4 with RbOH) and was continuously perfused at 200 ml hour−1 during the experiment. For voltage-dependent activation, currents were elicited by voltage steps from −100 to +100 mV, from a −80-mV holding potential. For voltage-dependent inactivation, currents were elicited by prepulse voltage steps from −50 to +90 mV from a −80-mV holding potential, each step being followed by a test pulse at +100 mV. All electrophysiology data were analyzed using Clampfit 10.7 (Molecular Devices).

**Molecular dynamics**

**Simulation setup**

Initial K2P2.1 (TREK-1) simulations in the absence of ML335 were initiated from PDB:5VK5. Later simulations were based on PDB:6CQ6 (20), which is indistinguishable from PDB:5VK5 except for a minor difference in the C-terinal portion of M4. Simulations in complex with ML335 were constructed from PDB:6W8C. In both cases, models consisted of residues 35 to 321, a disulfide bond was formed between C93 in one subunit with C93 in the other, missing loops were built with RosettaRemodel (87), and N and C termini were capped with methylamide and acetyl groups, respectively. All residues were assigned their standard protonation states at pH 7. Structures were embedded in pure 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) or POPC + 4% inner leaflet mole fraction 18:0-bisphosphate (18:0-20:4) PIP2 bilayers using CHARMM-GUI (22°C) and used standard recording solution at pH 7.4 supplemented with 10 mM Hepes (pH 7.2 with NaOH) and was continuously perfused at 200 ml hour−1 during the experiment. For voltage-dependent activation, currents were elicited by voltage steps from −100 to +100 mV, from a −80-mV holding potential. For voltage-dependent inactivation, currents were elicited by prepulse voltage steps from −50 to +90 mV from a −80-mV holding potential, each step being followed by a test pulse at +100 mV. All electrophysiology data were analyzed using Clampfit 10.7 (Molecular Devices).

**Devices**

**Patch clamp electrophysiology**

Human embryonic kidney cells (HEK293) were grown at 37°C under 5% CO2 in a Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 10% l-glutamine, and antibiotics (penicillin (100 IU ml−1) and streptomycin (100 mg ml−1)). Cells were transfected (in 35-mm-diameter wells) using Lipofectamine 2000 (Invitrogen) and a pIRE-GFP (Invitrogen) plasmid vector into which the gene encoding for mouse K2P2.1 (TREK-1) wild type or mutants has been inserted in the first cassette (4). DNA (1 μg) was used for K2P2.1 (TREK-1) and Loop2-sym-6, whereas 3 μg of DNA was necessary to record reliable currents from E234Q and Y270F. Data acquisition was performed using pCLAMP 10 (Molecular Devices) and an Axopatch 200B amplifier (Molecular Devices).

The inside-out configuration of the patch clamp technique was used to record K+ or Rb+ currents at room temperature (23°C ± 2°C) 24 to 48 hours after transfection (5, 20). Pipettes were pulled from borosilicate glass capillaries (TW150F-3, World Precision Instruments) and polished (MF-900 microforge, Narishige) to obtain 1- to 2-MΩ resistances.
bonds to carboxylate or carbonyl oxygens was determined on the time the ion crossed the midplane before exit from the cylinder. PCA by the plane separating S2 to S3 sites) exited the top (bottom) of an ion originating below (above) the midplane of the filter (defined on the selectivity filter, and a permeation event was recorded when an ion in the selectivity filter placed at the S2, S3, or S4 site. The force fields used for protein, lipids, water, and ML335 were CHARMM36m (89), CHARMM36 (90), TIP3P (91), and CGenFF 3.0.1 (92-94), respectively. Standard CHARMM parameters were used for ions (95).

Simulation details
Production data were collected on two platforms: Anton2 (96) at the Pittsburgh Supercomputing Center and local graphical processing unit (GPU) resources using GROMACS 2018 (97) (see table S3 for a full list). All systems were energy minimized for 8000 steps with 5 kcal/mol per Å² harmonic restraints on all protein heavy atoms, followed by a multistep equilibration in which protein restraints were gradually reduced over 10 to 12 ns. Next, for systems simulated under a membrane potential, we performed a 10-mV voltage jump every 5 ns until reaching 40 mV using the constant electric field protocol. For low [K+] simulations, solution ions were preincubated with waters at either S1/S3 or S2/S4, all K+, or all K+ with empty S3. Experiments were nonrandomized and nonblinded, and no pre-specified sample size was estimated. Measurements were taken from distinct samples. All data are presented as means ± SEM, and all experiments were repeated from N ≥ 2 different batches to mitigate biological variability. The number of experiments (n) as technical replicates is indicated in the figure legends. Significances are indicated in the figures using the following symbols: “*”, P < 0.05; “**”, P < 0.01; and “N.S.,” not statistically different.

Supplementary Materials
Supplemental material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/4/eaab9174/DC1

View request a protocol for this paper from Bio-protocol.

References and Notes
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and crystallized the proteins, collected diffraction data, and determined the structures. R.D. and A.W. collected anomalous diffraction data. M.L., A.M.N., F.A.-A., and, D.C. performed functional studies. F.A.-A. carried out and analyzed single-channel recordings. A.M.N., S.C., J.M.R., and M.G. designed and executed the simulations. M.L., A.M.N., F.A.-A., D.C., M.G., and D.L.M. analyzed the data. M.G. and D.L.M. provided guidance and support. M.L., A.M.N., F.A.-A., M.G., and D.L.M. wrote the paper. Competing interests: The authors declare that they have no competing interests. Materials and correspondence: Correspondence should be directed to M.G. or D.L.M. Requests for materials should be directed to D.L.M. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Coordinates and structures factors are deposited in the RCSB and will be released immediately upon publication. Additional data related to this paper may be requested from the authors.

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K2p channel C-type gating involves asymmetric selectivity filter order-disorder transitions


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Supplementary Materials for

K₂P channel C-type gating involves asymmetric selectivity filter order-disorder transitions


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The PDF file includes:

Figs. S1 to S10
Legends for movies S1 to S4
Tables S1 to S3
References

Other Supplementary Material for this manuscript includes the following:

(available at advances.sciencemag.org/cgi/content/full/6/44/eabc9174/DC1)

Movies S1 to S4
Fig. S1 K$_{2p}$ channel selectivity filters structure comparison. 

a, Superposition of the selectivity filters and permeant ions for: K$_{2p}$.1 (TREK-1) (6CQ6)(20) (smudge), K$_{2p}$.2.1 (TREK-1):ML335 (6CQ8)(20) (deep salmon), K$_{2p}$.2.1 (TREK-1):ML402 (cyan) (6CQ9)(20); K$_{2p}$.10.1 (TREK-2) (4BW5)(21) (pink), (4XDJ)(21) (magenta), (4XDK)(21) (purple); K$_{2p}$.4.1 (TRAACK) (3UM7)(22) (aquamarine), (4I9W)(23) (limon), (4WFE) (forest green)(24), (4WFF) (white)(24),(4WFG) (grey)(24), (4WFH) (black)(24); K$_{2p}$.4.1 (TRAACK) G124I (4RUE) (blue)(25) K$_{2p}$.4.1 (TRAACK) W262S (4RUF) (lime green)(25); K$_{2p}$.1.1 (TWIK-1) (3UKM)(26) (red). K$_{2p}$.3.1 (TASK-1) (6RV2) (orange)(43), K$_{2p}$.3.1 (TASK-1):BAY1000493 (6RV3) (yellow orange)(43), and K$_{2p}$.3.1 (TASK-1):BAY2341237(6RV4) (olive)(43). SF1, SF2 and ion binding positions, S1-S4, are indicated. Ions are shown as spheres and colored according to the parent structure. 

b, K$_{2p}$ channel structures, permeant ion concentration in crystallization conditions, and RMSD for all selectivity filter backbone atoms relative to K$_{2p}$.2.1 (TREK-1) (6CQ6)(20). Structures with two RMSD values indicate
structures having chains A/B and C/D, respectively. '*' indicates samples where permeant ions were part of the protein sample buffer.
Fig. S2 $K_{2p2.1}$ (TREK-1) selectivity filter potassium-dependent conformational changes. a, SF2 exemplar 2Fo-Fc electron density ($1_{\sigma}$) for $K_{2p2.1}$ (TREK-1) (left) and $K_{2p2.1}$ (TREK-1):ML335 (right) structures under 0 mM (pale yellow; blue white), 1 mM (yellow; pale cyan), 10 mM (light orange;
aquamarine), 30 mM (yellow orange; light blue), 50 mM (bright orange; marine), 100 mM (olive; slate), and 200 mM (orange; deep blue) \([K^+]\). Dashed lines indicate regions of disorder. Resolution and select residues and channel elements are indicated. b, and c Exemplar SF1-M2 loop density for b, \(K_{\text{p}2.1}\) (TREK-1) under 30 mM (yellow orange) and 200 mM (orange) \([K^+]\) and c, \(K_{\text{p}2.1}\) (TREK-1):ML335 under 0 mM (blue white) and 200 mM (deep blue) \([K^+]\). Examples show that the highest resolution low \([K^+]\) structure, 30 mM \([K^+]\), has a well-defined SF1-M2 loop, contrasting the poorly resolved SF2-M4 loop. Hence, the structural changes are local and not related to resolution.
Fig. S3

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(a) Structures of K_{\text{TREK-1}} (TREK-1):ML335 in the presence of different concentrations of K^+ (0 mM, 1 mM, 10 mM, 30 mM, 50 mM, 100 mM, 200 mM) for SF1 and SF2.

(b) Plot showing the root mean square deviation (RMSD) of K_{\text{TREK-1}} (TREK-1):ML335 against mM [K^+] for different structures.
Fig. S3 Selectivity filter structural changes as a function of potassium concentration. a, $K_{2p}2.1$ (TREK-1) and the $K_{2p}2.1$ (TREK-1):ML335 complex SF1 and SF2 structures determined at the indicated potassium concentrations: 0 mM (pale yellow; blue white), 1 mM (yellow; pale cyan), 10 mM (light orange; aquamarine), 30 mM (yellow orange; light blue), 50 mM (bright orange; marine), 100 mM (olive; slate), and 200 mM [K$^+$] (orange; deep blue). $K_{2p}2.1$ (TREK-1) panels show an overlay with the 200 mM [K$^+$] $K_{2p}2.1$ (TREK-1) structure in lighter shading. Labels indicate the last visible residue at points where the chain becomes disordered. Potassium ions from the 200 mM [K$^+$] structures are shown in all panels as a reference. b, $K_{2p}2.1$ (TREK-1) and $K_{2p}2.1$ (TREK-1):ML335 complex RMSD$_{Ca}$ as a function of [K$^+$]. Structures are compared to $K_{2p}2.1$ (TREK-1) in 200 mM [K$^+$] (PDB:6CQ6) and $K_{2p}2.1$ (TREK-1):ML335 complex in 200 mM [K$^+$] (PDB:6CQ8), respectively. Channel elements are grouped as follows: Core: residues 50-146, 153-255, 269-311; SF1-M2: residues 142-188; and SF2-M4: residues 251-295.
Fig. S4 Omit maps showing $K_{2P.2.1}$ (TREK-1) selectivity filter ion occupancy as a function of $[K^+]$. a,b, Polder omit maps(28) for structures of $K_{2P.2.1}$ (TREK-1) determined in 0 mM $[K^+]$ (pale yellow) (5σ), 1 mM $[K^+]$ (yellow) (4σ), 10 mM $[K^+]$ (light orange) (5σ), 30 mM $[K^+]$ (yellow orange) (4σ), 50 mM $[K^+]$ (bright orange)(5σ), 100 mM $[K^+]$ (olive) (4σ), and 200 mM $[K^+]$ (orange)(4σ) (a) or $K_{2P.2.1}$ (TREK-1):ML335 determined in 0 mM $[K^+]$ (blue white) (4σ), 1 mM $[K^+]$ (pale cyan) (4σ), 10 mM $[K^+]$ (aquamarine) (4σ), 30 mM $[K^+]$ (light blue) (4σ), 50 mM $[K^+]$ (marine) (4σ), 100 mM $[K^+]$ (slate) (4σ) and 200 mM $[K^+]$ (deep blue) (4σ) (b). Potassium ions are magenta spheres. Sites S1-S4 are labeled. ML335 is shown as sticks. SF1 in the 200 mM $[K^+]$ conformation is shown for all panels. Select residues are indicated.
Fig. S5 Water interactions with the selectivity filter. a, Final frame of High $[K^+]$ +40 mV/ML335 simulation 4, showing SF1 (left) and SF2 (right). b, Final frame of High $[K^+]$/+40 mV simulation 12. c, Final frame of High $[K^+]$/+40 mV simulation 21. d, Final frame of Low $[K^+]$/0 mV simulation 29. In all panels, water molecules interacting with the extracellular face of the selectivity filter are shown as spheres. Water oxygen
atom occupancy maps calculated from the simulation data are shown as red mesh and are contoured at the same level (density contains voxels with occupancy >7%) in all panels. Maps in a and d were calculated from all High [K⁺]/+40 mV/ML335 and Low [K⁺]/0 mV simulation trajectories, respectively. Map in b and c was calculated from all High [K⁺]/+40 mV simulation trajectories. c and d show examples where filters have become disordered.
**Fig. S6**

**K₂P₂.1 (TREK-1) principal component analysis (PCA).**

- **a, b:** PCA projection of selectivity filter conformations for SF1 (a) and SF2 (b). Grey points indicate K₂P₂.1 (TREK-1) conformations from all simulations in this work; black points represent projections of SF1 and SF2 conformations obtained from other studies.

- **c:** Representation of various Kₐ channels and their mutations.

- **d:** Detailed clusters and conformations for SF1 and SF2, highlighting different states and transitions.

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the Fig. S1b $K_{2P}$ crystal structures. c, Representative non-$K_{2P}$ selectivity filter conformations and their projections into PCA space. Grey points indicate $K_{2P}2.1$ (TREK-1) conformations from all simulations (SF1 and SF2). Lines and colored points show the PCA projected location of SF conformations from the indicated tetrameric potassium channel crystal structures: KcsA closed/conductive (1K4C) and closed/inactivated (1K4D)(14) (firebrick); Kv1.2-2.1 chimera (2R9R)(33) and V406W mutant (5WIE)(17) (chartreuse); NaK (2AHZ)(36) and K$^+$ selective mutant NaK2K (3OUF)(27) (slate); and KcsA E71A open/conductive (5VK6) and open/inactivated (5VKE)(15) (orange). d, Hierarchical clustering of SF conformations from all High [K$^+$] simulations. Clustering was performed on PC1-3, for clarity only PC1 and PC2 are shown. Points representing selectivity filter conformations in PCA space are colored according to their membership in one of 14 identified clusters. A single representative conformation is shown for each cluster, with the exception of the native state (cluster 0) for which representative conformations from both SF1 and SF2 are shown. For each representative conformation intact ion binding sites are indicated with dotted circles.
Fig. S7 $K_{\text{2P}}$ channel pore domain comparisons. a, $K_{\text{2P}}$ 2.1 (TREK-1):ML335 complex (white) with a view showing the SF1-M2 loop. SF1-M2 loop (red) and SF2-M4 loop (blue) are indicted. ML335 (black) is shown in sticks with a transparent surface. b, Sequence alignment of PD1 for the indicated channels. P1 and M2 helices (blue), SF1 (orange), and SF1-M2 loop (red) are indicated. Terminal residue of the selectivity filter is highlighted. Arrows denote the boundaries of the SF1-M2 loop. c, $K_{\text{2P}}$ 2.1 (TREK-1):ML335 complex.
(white) with a view showing the SF2-M4 loop. SF1-M2 loop (red) and SF2-M4 loop (blue) are indicted. ML335 (black) is shown in sticks with a transparent surface. d, Sequence alignment of PD2 for the indicated channels. Conserved residues are shaded in slate. Dashed red and blue boxes indicate the SF1-M2 (c) and SF2-M4 loops (d). Conserved selectivity filter N/D is shaded orange. Pro150, Ala259, and equivalents are shaded red. e, Per-frame probability of finding a particular number of hydrogen bonds to the Glu234 sidechain carboxylate in all K$_{2P}$2.1 (TREK-1) simulations. Dotted lines indicate the overall average number of hydrogen bonds calculated for each simulation condition. f, Per-frame probability of a hydrogen bond between the indicated groups in all K$_{2P}$2.1 (TREK-1) simulations. g, Sequence alignment of M1 for the indicated channels. Conserved glutamate is highlighted red.

Sequences (b,d, and g) are from human K$_{2P}$ channels: K$_{2P}$2.1 (TREK-1) AAD47569.1, K$_{2P}$10.1 (TREK-2) NP_612190.1, K$_{2P}$4.1 (TRAAK) AAI10328.1, K$_{2P}$3.1 (TASK-1) NP_002237.1, K$_{2P}$9.1 (TASK-3) NP_001269463.1, K$_{2P}$5.1 (TASK-2) NP_003731.1, K$_{2P}$1.1 (TWIK-1) NP_002236.1, K$_{2P}$6.1 (TWIK-2) NP_004814.1, K$_{2P}$16.1 (TALK-1) NP_115491.1, K$_{2P}$17.1 (TALK-2) AAK28551.1, K$_{2P}$12.1 (THIK-2) NP_071338.1, K$_{2P}$13.1 (THIK-1) NP_071337.2, K$_{2P}$15.1 (TASK-5) EAW75900.1, K$_{2P}$18.1 (TRESK) NP_862823.1. SF1 and SF2 sequence and numbers for K$_{2P}$2.1 (TREK-1)$_{crys}$ (PDB:6CQ6)(20) are identical to that of K$_{2P}$2.1 (TREK-1) AAD47569.1.
**Fig. S8**

**Fig. S8 K$_{2P}$ patch clamp recordings.** a, Exemplar current traces from inside-out membrane patches of HEK293 cells expressing K$_{2P}2.1$ (TREK-1), K$_{2P}2.1$ (TREK-1) E234Q, K$_{2P}2.1$ (TREK-1) Y270F, or K$_{2P}2.1$ (TREK1) Loop2Sym6, in 150 mM K$^+$[ext]/150 mM Rb$^+$[int]. Inset shows voltage protocol. b, Rectification coefficients ($I_{+100mV}/I_{-100mV}$) calculated from currents recorded on $n \geq 5$ individual patches. * $p < 0.05$ compared to K$_{2P}2.1$ (TREK-1). N.S, not statistically different. c, Exemplar current traces from inside-out membrane patches of HEK293 cells expressing K$_{2P}2.1$ (TREK-1) or K$_{2P}2.1$ (TREK-1) E234Q in response to an inactivation protocol (inset). d, Channel availability curves determined by plotting the normalized peak currents ($I/I_{-50mV}$) measured at $+100$ mV as a function of pre-pulse voltages ($n \geq 4$). For panels b, and d, data represent mean ± s.e.m.
Fig. S9 Activation alters $K_{2P.2.1}$ (TREK-1) mutant ion selectivity. a-c, Exemplar two-electrode voltage clamp (TEVC) recordings of $K_{2P.2.1}$ (TREK-1) (a), $K_{2P.2.1}$ (TREK-1) E234Q (b), and $K_{2P.2.1}$ (TREK-1) Y270F (c) at 20°C (left) and 30°C (right) in solutions of 96 mM Na$^+$/2 mM K$^+$ (black) or 96 mM N-methyl-D-glucamine/2 mM K$^+$ (grey). d, and e, Potassium selectivity recorded in Xenopus oocytes in $K^+/Na^+$ solutions (98.0 mM total) at pH$_o$ = 7.4 at d, 20°C and e, 30°C (n=6). Data are background subtracted using uninjected oocytes. Grey line represents Nernst equation $E_{\text{rev}} = \frac{RT}{F} \times \log([K^+]_o/[K^+])$, where $R$ and $F$ have their usual thermodynamic meanings, $z$ is equal to 1, and $T = 20°C$ or 30°C, assuming $[K^+] = 108.6$ mM ((110)). f, $E_{\text{rev}}$ as a function of temperature for the indicated channels from TEVC experiments in Xenopus oocytes. g, $E_{\text{rev}}$ at 0 and -50 mmHg measured from inside-out membrane patches of HEK293 cells expressing the indicated channels. * p < 0.05 compared to $K_{2P.2.1}$ (TREK-1) at the same pressure.
N.S., not statistically different. (n ≥ 4). Grey indicates statistical significance between the 0 mM and -50 mM Hg measurements. For panels (d)-(g), data represent mean ± s.e.m.
Fig. S10 M3 glutamate network destabilization facilitates $K_{2p3.1}(TASK-1)$ C-type gate closure. 

**a,** Comparison of the SF2-M4 loop and surrounding elements $K_{2p3.1}$ (TASK-1) (PDB: 6RV2)(43) (yellow) and $K_{2p2.1}$ (TREK-1) (PDB:6CQ8)(20) (transparent purple). Key network residues are shown as sticks and are labeled. Dashed lines show hydrogen bonds. **b-d,** Exemplar two-electrode voltage clamp (TEVC) recordings of **b,** $K_{2p3.1}$ (TASK-1), **c,** $K_{2p3.1}$ (TASK-1) E182Q, and **d,** $K_{2p3.1}$ (TASK1) Y220F at pH$_{ext}$: 9.0 (dark blue), 8.1 (blue), 7.8 (light blue), 7.4 (grey), 7.1 (pink), 6.5 (red), 5.9 (maroon). **e,** pH activation curves for $K_{2p3.1}$ (TASK-1) (black) and $K_{2p3.1}$ (TASK1) Y220F (blue), showing the fraction of the current at 0 mV at each pH relative to the current at pH 9.0 (n≥10). Data in 'e' represent the mean ± s.e.m. (n≥10).
Supplementary Movie 1 High [K\(^{+}\)]/+40 mV simulation trajectory. Pore helix 1 and SF1-M2 loop are shown in cartoon representation, SF1 is shown as sticks, and potassium ions are spheres of varying colors. Movie represents the first 1920 ns of simulation 1.

Supplementary Movie 2 Low [K\(^{+}\)]/0 mV simulation trajectory. Pore helix 2 and SF2-M4 loop are shown in cartoon representation, SF2 is shown as sticks, M4 is shown as transparent cartoon, and the potassium ion is shown as a magenta sphere. Residues involved in the Glu234 network are shown as green sticks. Movie represents the first 1920 ns of simulation 29.

Supplementary Movie 3 Morph between the SF1 active and inactive conformations. 0 mM [K\(^{+}\)]:ML335 structures and 1 mM [K\(^{+}\)] structures represent the active and inactive conformations, respectively. Selectivity filter is yellow orange. Asn147 and Thr142 are shown as sticks. Potassium ions are magenta spheres.

Supplementary Movie 4 Morph between the SF2 active and inactive conformations. 0 mM [K\(^{+}\)]:ML335 structures and 1 mM [K\(^{+}\)] structures represent the active and inactive conformations, respectively. Selectivity filter is yellow orange. Asp256 and Thr251 are shown as sticks. Potassium ions are magenta spheres.
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a Values in parentheses are for highest-resolution shell.
Each data set is from a single crystal.
Anomalous peak heights (in $\sigma$) from long-wavelength data above ($\lambda = 3.35$ Å) the potassium K-edge as calculated with ANODE\cite{86} based on K$_{2p}$2.1 (TREK-1) (6CQ6\cite{20}).

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REFERENCES AND NOTES


70. Y. Kim, C. Gnatenco, H. Bang, D. Kim, Localization of TREK-2 K\(^+\) channel domains that regulate channel kinetics and sensitivity to pressure, fatty acids and pH\(_i\). *Pflugers Arch.* **442**, 952–960 (2001).


