INTRODUCTION

K_{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_{2p}s is unknown. Here, combining K_{2p}2.1 (TREK-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_{2p} C-type gating. These asymmetric order-disorder transitions, enabled by the K_{2p} heterodimeric architecture, encompass pinching and dilation, disrupt the S1 and S2 ion binding sites, require the uniquely long K_{2p} SF2-M4 loop and conserved “M3 glutamate network,” and are suppressed by the K_{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_{2p} channel modulator development.

RESULTS

Potassium-dependent selectivity filter structural changes

Despite the fact that C-type gating is the principal K_{2p} gating mechanism (3–6) and that previously determined K_{2p} structures show major conformational changes that affect function (20–26), all prior
K₂P 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 K₂P2.1 (TREK-1) construct, K₂P2.1<sub>425-271</sub> (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<sub>253</sub>-Lys<sub>271</sub>) (figs. S2 and S3). Additional changes were observed in SF1 residues Gly<sub>144</sub>-Asn<sub>147</sub> 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 K₂P2.1 (TREK-1) activator ML335 (20) at resolutions of 3.4, 2.6, 3.0, 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 K₂P2.1 (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 K₂P2.1 (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 = 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 K₂P 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 K₂P2.1

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**Fig. 1. K₂P2.1 (TREK-1) selectivity filter potassium-dependent conformational changes.** (A) Exemplar 0 and 200 mM [K⁺] K₂P2.1 (TREK-1) SF2 2Fo-Fc electron density (1σ). Select residues and channel elements are indicated. Dashes indicate disordered regions. (B) [K⁺]-dependent structural changes in K₂P2.1 (TREK-1) SF1 (left) and SF2 (right). Top: Superpositions of structures determined in 0 (pale yellow), 1 (yellow), 10 (light orange), 30 (yellow orange), 50 (bright orange), 100 (olive), and 200 (orange) mM [K⁺]. Bottom: Superposition of 0 and 200 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 K₂P2.1 (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).

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**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 K₂P2.1
(TREK-1). Initially, we simulated two conditions: (i) 180 mM $[\text{K}^+]$ and a +40-mV applied membrane potential (denoted “High $[\text{K}^+]$/+40 mV,” 36.5 μs aggregate) and (ii) the same $[\text{K}^+]$ and potential with bound ML335 (denoted “High $[\text{K}^+]$/+40 mV/ML335,” 31.6 μs aggregate). Both conditions showed many permeation events (144 and 253 for High $[\text{K}^+]$/+40 mV and High $[\text{K}^+]$/+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 $[\text{K}^+]$/+40 mV/ML335 trajectories (8 of 10) remained in a stable, ion-conducting state. By contrast, most (7 of 12) of the High $[\text{K}^+]$/+40 mV trajectories entered long-lived (>1 μs) nonconducting states from which they did not recover and that were characterized by obvious disruptions of the initial selectivity filter conformation. Concordantly, the two conditions had a substantial difference in the current (0.3 pA versus 1.3 pA for High $[\text{K}^+]$/+40 mV and High $[\text{K}^+]$/+40 mV/ML335, respectively) (Fig. 3B). There were no major changes during the simulations in the M4 helix position or in other parts of the channel outside of the selectivity filter when compared with their starting positions as defined by the crystal structures.

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 homotetrameric channel KcsA (31), we first characterized the role that water molecules have on the K$_{2P2.1}$ (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 amides of Phe$_{145}$/Gly$_{146}$ and Phe$_{254}$/Gly$_{255}$, respectively (fig. S5, A and B). As the K$_{2P2.1}$ (TREK-1) filter moves away from the canonical, conductive conformation, these well-organized networks dissolve (fig. S5C).

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 K$_{2P2.1}$ (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\(_{\text{2P}}\)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\(^{260}\) 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 conduction 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 x-ray structures (Fig. 2) into a new coordinate system wherein the greatest variance in conformations lies along the first axis (principal 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\(_{\text{2P}}\) 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 both cases, ML335 increases channel open probability but not the single-channel conductance (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\(_{\text{2P}}\) 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\(_{\text{2P}}\) 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-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 K2P modulator pocket, is central to C-type gate activation of K2Ps (20).

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

In most potassium channels, including the first K2P 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). K2Ps 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 K2P 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-Gly155 from PD1 onto PD2, denoted “Loop2 Sym-6” (Fig. 5B). 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 to the outer transmembrane helix of the pore domain, 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, given the 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-Gly375 from PD1 onto PD2, denoted “Loop2 Sym-6” (Fig. 5B). Loop2 Sym-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, Loop2 Sym-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), Loop2 Sym-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 Loop2 Sym-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.

**Fig. 4. Effects of ML335 on K2P2.1 (TREK-1).** (A) Exemplar K2P2.1 (TREK-1) single-channel recordings at −100 mV before (left) and after (right) application of 30 μM ML335 to the same cell-attached patch. (B) Exemplar K2P2.1 (TREK-1) single-channel recordings at −100 mV in the presence of 5 μM ML335 applied in the pipette solution of a cell-attached patch. (C) Open channel probability at −100 mV from single-channel analysis calculated on recordings of ≥30-s duration. (D and E) Single-channel amplitude at (D) −100 mV and (E) +50 mV. Error bars indicate SEM (n = 5 to 7). ** indicates P < 0.01 and “N.S.” indicates not statistically different relative to K2P2.1 (TREK-1).
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
Further, as K$_2$P heterodimer formation yields channels having S4 sites and can be converted into a potassium-selective channel by the nonselective bacterial channel NaK, which has only the S3 and structure compromises ion selectivity are reminiscent of studies of 48–56 provides a mechanism for the emergence of heterodimer properties together with the two nonmutually exclusive inactivation modes likely two unique SF1-M2 and SF2-M4 loops, this structural diversification for K$_2$P 2.1-(TREK-1) (46) 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$_2$P 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$_2$P PD1 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$_2$P21 (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$_2$P family (fig. S7D). Disruption of the M3 glutamate network blunts responses to diverse stimuli in distantly related K$_2$P (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$_2$P except K$_2$P18.1 (TRESK), the only K$_2$P 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$_2$P3.1 (TASK-1), E182K, disrupts function (63) underscores the importance of the M3 glutamate network and SF2-M4 loop in gating throughout the K$_2$P family.

Our studies establish that K$_2$P 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$_2$P 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$_2$P 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 ideas that the K2P selectivity filter and its supporting architecture are dynamic under basal conditions (20), that ion permeation requires limiting filter mobility through ligand binding to the K2P modulator pocket or by conformational changes transmitted through the M4 helix (20), that permeant ions organize and stabilize the K2P 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 findings corroborating the ideas that the K2P selectivity filter and its supporting architecture are dynamic under basal conditions (20). The key role for the SF2-M4 loop in transducing gating cues sensed by intracellular channel components to the K2P 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 K2P 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 K2P 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 K2P loop for selective small molecule or biologic modulators directed at K2P-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 K2P2.1 (TREK-1), denoted K2P2.1crys, 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 His10 tag was expressed and purified from *Pichia pastoris* as previously described (20).

**Crystallization and refinement**

Purified K2P2.1crys 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 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-thioglucoiside (HTG), 0.02% cholesteryl 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 bound to the K2P2.1crys:ML335 complex, 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 pH₂ 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).

Datasets for K2P2.1crys 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 K2P2.1crys 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 K2P2.1crys:ML335 complex 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 K2P2.1crys 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 K2P2.1crys 0, 1, 10, and 50 mM structures; whereas, four potassium ions were modeled into 2Fo-Fc densities of the Apo K2P2.1crys 100 and 200 mM structures. Four potassium ions were modeled for all the K2P2.1crys: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 K2P2.1crys 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 K2P2.1crys 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 pH₂ 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° 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

$$A = A_{\text{min}} + (A_{\text{max}} - A_{\text{min}}) / (1 + e^{(T - T_{1/2})/H})$$

where $A_{\text{min}}$ and $A_{\text{max}}$ are the minimum and maximum activation, respectively, $T_{1/2}$ is the half maximal inhibitory concentration of extracellular protons, and $H$ 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

$$A = A_{\text{min}} + (A_{\text{max}} - A_{\text{min}}) / (1 + ([H^+]_o/K_{1/2})^S)$$

where $A_{\text{min}}$ and $A_{\text{max}}$ are the minimum and maximum activation, respectively, $K_{1/2}$ is the half maximal inhibitory concentration of extracellular protons, 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 pIRES-GFP (Invitrogen) plasmid vector into which the gene encoding for mouse K$_{\text{2P}}$2.1 wild type or mutants has been inserted in the first cassette (4). DNA (1 µg) was used for K$_{\text{2P}}$2.1 (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° ± 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 K$_{\text{2P}}$2.1 (TREK-1) and mutants was performed by applying a −50-mmHg pressure to the inside-out patch through a high-speed pressure clamp (HSPC-1, ALA Scientific Instruments) connected to the electrode suction port, after recording the current at 0 mmHg. Pipette solution contained 150 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 2 mM MgCl$_2$, and 20 mM Hepes (pH 7.4 with NaOH). Bath solution contained 145 mM KCl, 3 mM MgCl$_2$, 5 mM EGTA, and 20 mM Hepes (pH 7.2 with KOH) and was continuously perfused at 200 ml hour$^{-1}$ during the experiment. K$_{\text{2P}}$2.1 (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 (QF100-70-7.5, 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 (N Po, 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 K$_{\text{2P}}$2.1 (TREK-1) and mutants were recorded from inside-out patches. Pipette solution contained 150 mM KCl, 3.6 mM CaCl$_2$, and 10 mM Hepes (pH 7.4 with KOH). Bath solution contained 150 mM RbCl, 2 mM EGTA, and 10 mM Hepes (pH 7.4 with RbOH) and was continuously perfused at 200 ml hour$^{-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 K$_{\text{2P}}$2.1 (TREK-1) simulations in the absence of ML335 were initiated from PDB:5V5K. Later simulations were based on PDB:6CQ6 (20), which is indistinguishable from PDB:5V5K except for a minor difference in the C-terminal portion of M4. Simulations in complex with ML335 were constructed from PDB:6W8C. In both cases, models consisted of residues 35 to 321, an additional PIP$_2$ lipid per channel was solvated in 180 mM NaCl, 200 mM KCl, 5 mM MgCl$_2$, 2 mM CaCl$_2$, 5 mM EGTA, and 20 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 prepulse 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).
were sampled from the trajectories every 480 to 500 ps. All analyses were performed using a Shapiro-Wilk test, followed by an equality of variances test using a Levene’s test. For samples with similar variances, significance was evaluated using either a paired or unpaired Student’s t test. For data that were not normally distributed, a nonparametric Mann-Whitney test or a Wilcoxon signed-rank test for paired analyses was used. For samples with unequal variance, significance was evaluated with a Welch’s t test.

Experiments were nonrandomized and nonblinded, and no prespecified 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.

**SIMPLIFIED MATERIALS**

Supplemental material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/4/eabc9174/DC1

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

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**Statistical tests**

For datasets where significance is indicated, a normality test was performed using a Shapiro-Wilk test, followed by an equality of variances
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I. Introduction

II. Material and Methods

III. Results

IV. Discussion

V. Conclusion

Acknowledgments

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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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