Reviewed Preprint
v1 • November 14, 2024
Not revised

Reviewed Preprint
v2 • March 11, 2026
Revised by authors

For correspondence:
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Competing interests: No competing interests declared

Funding: See page 16

Reviewing editor: Jon T Sack, University of California, Davis, United States

Distinct mechanisms of inhibition of Kv2 potassium channels by tetraethylammonium and RY785

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eLife Assessment

This study represents an **important** advance in our understanding of how certain inhibitors affect the behavior of voltage gated potassium channels. Robust molecular dynamics simulation and analysis methods lead to a new proposed inhibition mechanism with strength of support being mostly **convincing**, though computational evidence is limited for some conformations discussed. This study has considerable significance for the fields of ion channel physiology and pharmacology and could aid in development of selective inhibitors for protein targets.

https://doi.org/10.7554/eLife.101855.2.sa3

Abstract

Voltage-gated K^+ channels play central roles in human physiology, both in health and disease. A repertoire of inhibitors that are both potent and specific would therefore be of great value, not only as pharmacological agents but also as research tools. The small molecule RY785 has been described as particularly promising in this regard, as it selectively inhibits channels in the Kv2 subfamily with high potency. Kv2 channels are expressed in multiple cell types in humans, and are of particular importance for neuronal function. The mechanism of action of RY785 has not yet been determined at the molecular level, but functional studies indicate it differs from that of less specific inhibitors, such as quaternary-ammonium compounds or aminopyridines; RY785 is distinct also in that it is electroneutral. To examine this mechanism at the single-molecule level, we have carried out a series of all-atom molecular dynamics simulations based on the experimental structure of the Kv2.1 channel in the activated, open state. First, we report a 25-microsecond trajectory calculated in the absence of any inhibitor, under an applied voltage of 100 mV, which demonstrates outward K^+ flow under simulation conditions at rates comparable to experimental measurements. Additional simulations in which either RY785 or tetraethylammonium (TEA) is introduced in solution show both inhibitors spontaneously enter the channel through the cytoplasmic gate, with distinct effects. In agreement with prior structural studies, we observe that TEA binds to a site adjacent to the selectivity filter, on the pore axis, thereby blocking the flow of K^+ ions. RY785, by contrast, binds to the channel walls, off-axis, and allows K^+ flow while the cytoplasmic gate remains open. The observed mode of RY785 binding, however, indicates that its mechanism of action is to stabilize and occlude a semi-open state of the gate, by bridging hydrophobic protein-protein interactions therein; this hypothesis would explain the puzzling experimental observation that RY785 recognition influences the gating currents generated by the voltage sensors, 3 nm away.

Introduction

Voltage-gated potassium (Kv) channels are an important class of membrane proteins involved in a variety of crucial physiological processes, both in health and disease. These channels contribute to confer electric excitability to certain types of cells, such as neurons and cardiomyocytes, by sensing and responding to changes in the transmembrane voltage. The nature of this response varies across different types of Kv channels, but in essence it entails a structural mechanism that results in the opening or closing of a pathway across the protein through which K^+ ions may flow at a fast rate. This mechanism has been the focus of numerous electrophysiological studies and structure-function analyses spanning several decades, and by now it appears well established (1-4).

As for other proteins of interest, inhibitors of Kv-channel function have been key to advance our understanding of these systems at the molecular level (5). Examples include non-specific blockers, such as quaternary-ammonium compounds (6) and aminopyridines (7); and polypeptide toxins isolated from animal venoms, such as stromatoxin-1 (8), hanatoxin (9) and guangxitoxin (10). A range of small-molecule inhibitors have also been identified (11), but translational applications have been hampered by their modest potency and poor ability to discriminate among different Kv-channel types. In a recent breakthrough, however, a high-throughput screening identified an inhibitor of Kv2 channels, known as RY785, which appears promising both in terms of specificity and potency (12). Subsequent electrophysiological studies of the mode of action of this compound, by Sack and coworkers (13), indicated that RY785 binds to a site in the interior of rat Kv2.1 channels that, at least in part, coincides with that used by tetramethyl-ammonium (TEA), a well-characterized cationic blocker (14-16). Access to this site requires voltage activation of the channel, i.e. opening of the ion-permeation pathway on its cytosolic side. Unlike TEA, though, RY785 is electroneutral, and so it is not immediately apparent whether it impedes K^+ flow directly or indirectly. Intriguingly, Sack and coworkers also report RY785 accelerates the deactivation of the voltage sensors (13), which lie over 3 nm away from the TEA binding site. This observation indicates RY785 recognition alters the conformational energetics of the channel, and that its mode of action, as yet unknown, is more complex than that of open-pore blockers such as TEA.

In parallel, Swartz and coworkers recently succeeded to determine an atomic-resolution structure of the channel domain of rat Kv2.1, uninhibited and immersed in a lipid nanodisc, using single-particle cryo-electron microscopy (17). Like other Kv channels, the structure shows Kv2.1 is a homotetramer, and that each subunit includes six trans-membrane alpha-helices (S1-S6). The ion pore is at the center of this complex, formed by assembly of the last two transmembrane helices in each subunit (and the connector in between); the extracellular half of this pore, or selectivity filter, is much narrower than the intracellular half, and features a series of K^+ binding sites in a configuration that indicates the filter is in a conductive state. Four voltage-sensing domains surround the pore, each formed by the first four helices in each subunit (17); an additional alpha-helix connects the sensor to the pore in each subunit, thereby coupling the voltage-response of the former to the opening/closing of the latter. Interestingly, the cryo-EM experiment resolved the channel structure in a unique conformation, namely one with the four voltage-sensors in the activated state; accordingly, the intracellular side of the pore domain is wide open. Thus, despite the fact that there is no voltage difference across the lipid nanodisc, the experimental condition somehow mimics the effect of membrane depolarization (i.e. positive-inside voltages), which causes this channel to be predominantly activated/open. To our knowledge, the structure of wild-type Kv2.1 in the deactivated/closed state remains unknown; however, Swartz and coworkers also determined a structure of a Kv2.1 mutant in which pore and voltage domains are seemingly decoupled (17). In this structure the sensors remain activated, and the selectivity filter is unchanged, but the intracellular side of the pore, or gate, is closed; specifically, a rearrangement of helices S5 and S6 causes a hydrophobic constriction to form at the position of residues Ile405 and Pro406, which seems too narrow to permit K^+ flux.

In this study, we leverage the discovery of the atomic structure of activated Kv2.1 to gain novel insights into the mode of inhibition of RY785. To do so, we employ atomically-detailed computer simulations of the recognition of this inhibitor by the channel, and quantify its effect on K^+ permeation, relative to the uninhibited channel; the results are compared with an analogous analysis of the mode of TEA binding and inhibition, which serve as a control. Taken together, our observations indicate that RY785 inhibits Kv2.1 function not by directly blocking K^+ currents, when the pore is open, but by facilitating the closure of its intracellular gate.

Results

Simulation of activated Kv2.1 provides realistic description of outward K^+ current

To establish a baseline for this investigation, we first examined the rate and mechanism of K^+ permeation through activated Kv2.1 in the absence of any inhibitors. To do so, we carried out an all-atom MD simulation of the channel embedded in a model phospholipid bilayer, under the influence of a transmembrane electric field (Fig. 1). The channel structure employed in this calculation was that recently determined by Swartz and co-workers using cryo-electron microscopy (17); this structure comprises the pore domain and four voltage sensors (Fig. 1), solubilized in a lipid nanodisc. To maximize the statistical significance of our results, the simulation was calculated using an Anton 2 supercomputer, which enabled us to generate a continuous 25-μs trajectory. The simulation was initialized with two K^+ ions inside the narrowest portion of the pore, known as selectivity filter, and no voltage difference across the membrane; after 500 ns, a voltage difference of 100 mV was introduced, positive on the inside. As shown in Fig. 2A, at this point K^+ ions begin to steadily flow across the channel in a single file and in an outward direction. In the observed mechanism, three K^+ ions concurrently reside in the filter, fluctuating in a concerted fashion among five transient binding sites therein, termed S_0, S_1, S_2, S_3 and S_4 (Fig. 2A, 2B). Arrival of an additional K^+ ion through the cytoplasmic gate at a site termed S_{cav}, near the center of a water-filled cavity adjacent to S_4, leads to a metastable 4-ion configuration. This configuration is short-lived, on account of the increased electrostatic repulsion between the K^+ ions, and the incoming K^+ ion often returns to the interior (not shown). An alternative outcome, fostered by the applied electric field, is the ejection of the outermost K^+ ion from the filter into the extracellular space. This event is immediately followed by the outward movement of the two other ions within the filter, and that in S_{cav}, thereby restoring the initial 3-ion configuration (Fig. 2B). Four iterations of this molecular cycle thus result in a complete ion permeation event. As has been noted elsewhere, water molecules can co-permeate with K^+ but do so rarely; in most of the observed permeation events, the ions become completely dehydrated near the center of the selectivity filter (18, 19).

In total, we observed 45 complete permeation events, i.e. approximately one K^+ ion traverses the channel from side to side every 0.5 μs. This high throughput rate appears to be explained by the fact that K^+ remains at near bulk density throughout the pore domain (Fig. 2C); as K^+ ions cross the channel, they encounter both free-energy wells and free-energy barriers, but it appears that the balance of intermolecular interactions therein is such that these features are relatively shallow, and therefore do not cause large density variations e.g. 100-fold or greater (Fig. 2C). Experimental estimates of the single-channel conductance of Kv2.1 range from 8 to 10 pS depending on the precise condition and type of measurement (20-22); by comparison, the permeation rate observed in our simulation translates into 3 pS. Given the myriad approximations and simplifications inherent to MD simulations, this result is very encouraging, and suggests our methodology results in an adequate description of the mechanism of ion conduction of this channel. We therefore posit, by extension, that the same simulation design should be appropriate to examine how this mechanism might be modulated by an inhibitor, as described below.

Figure 1. Structure and simulation of the Kv2.1 channel in the activated state.

(**A**) Structure of Kv2.1 determined by single-particle cryo-electron microscopy in a lipid nanodisc (17), viewed from the cell intracellular side and along the perpendicular to the membrane. The channel is an assembly of four identical subunits (in colors); the transmembrane ion pore is formed at the center of the assembly, and the voltage sensors are found in the periphery, in a domain-swapped configuration. A K^+ ion is shown in magenta, inside the selectivity filter. (**B**) Simulation system used in this study, comprising the channel (yellow), a phospholipid bilayer (gray), and a 300 mM KCl buffer (magenta, green). The figure depicts the final configuration of the 25-μs MD trajectory described in Figure 2. The total number of atoms is 201,954, some of which are omitted in the figure, for clarity. (**C**) Close-up of the pore domain and the selectivity filter. Note two protein subunits are omitted, for clarity. The configuration represented is that shown in panel (B); in this configuration, K^+ ions are found in sites S_1, S_3 and S_4 within the selectivity filter, while sites S_0, S_2 and S_{cav} are transiently vacant.

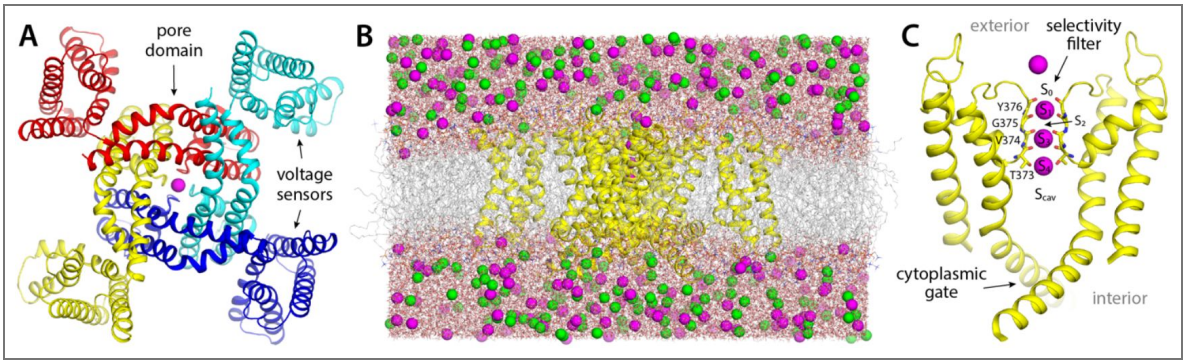
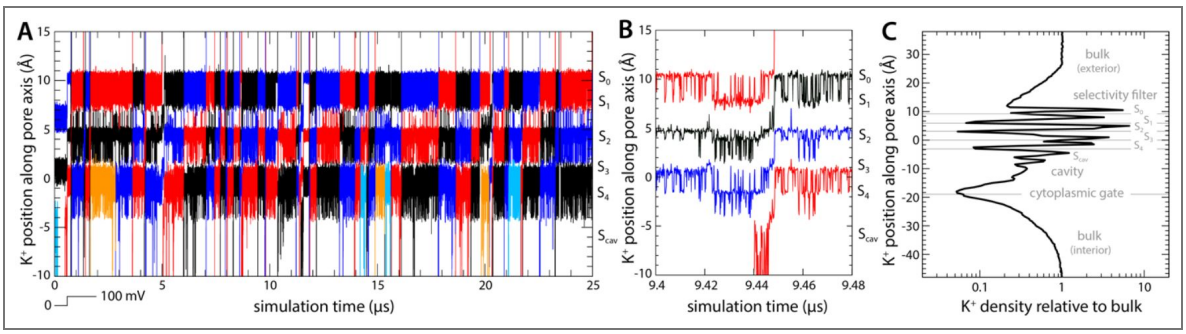


Figure 2. Mechanism of K^+ permeation through the Kv2.1 channel.

(**A**) Time traces of the position of K^+ ions along the central axis of the channel as they reach and permeate the selectivity filter towards the extracellular side (black, red and blue). Ions that do not reach the filter are not shown for clarity (those that reach the filter but return to the cytoplasmic side before permeating are shown in orange and cyan). The approximate location of each of the K^+ binding sites (S_0 through S_4, and S_{cav}) along the pore is indicated alongside the plot. (**B**) Same as (A), for a fragment of the trajectory that illustrates the knock-on mechanism that initiates and completes each of the observed permeation events. (**C**) Density for K^+ ions along the channel axis, relative to the bulk concentration value of 300 mM. Density peaks correspond to each of the K^+ binding sites within and adjacent to the selectivity filter. For reference, gray horizontal lines indicate the average position of selected protein atoms: in the selectivity filter these are, from top to bottom, the backbone carbonyl oxygens of residues Y376, G375, V374 and T373, and the sidechain hydroxyl oxygen of T373; and in the cytoplasmic gate, the alpha-carbon of residue P408. All ions in the simulation system contribute to this profile, but only while they reside in a cylindrical volume of diameter equal to 12 Å, centered in and parallel to the channel axis, extending across the whole system.



Spontaneous binding of tetraethylammonium blocks K^+ permeation

Tetraethylammonium (TEA) is a well-characterized non-specific K^+-channel blocker; it can inhibit K^+ currents completely, whether applied externally or internally, albeit with modest potency. For Kv2.1 in particular, the IC values are around 5 mM and 0.2 mM, respectively (23, 24). Structural and computational studies carried out two decades ago using the bacterial homolog KcsA as a model system revealed that the binding site for internal TEA (and related quaternary-ammonium blockers) is adjacent to S_{cav} (14, 15); from this finding it is logical to infer that internal TEA enters the pore through the cytoplasmic gate and that it precludes K^+ ions from reaching the selectivity filter.

To verify this putative inhibitory mechanism, which to our knowledge had not been directly demonstrated before, we carried out a simulation identical to that reported in the previous section, except that TEA was added in solution on the cytoplasmic side of the lipid membrane. The inhibitor was confined to explore a wide cylindrical volume co-axial with the pore but was otherwise permitted to diffuse freely. Under an applied voltage of 100 mV, however, TEA rapidly entered the channel interior through the cytoplasmic gate and did not return to the solution in a 5-μs trajectory (Fig. 3A, 3B). Once in the water-filled cavity under the selectivity filter, the inhibitor continues to be highly dynamic, but tends to reside in a site about 1.5 Å away from S_{cav} (Fig. 3C), and the nitrogen atom that carries the positive charge of the ammonium ion rarely deviates more than 2 Å from the pore axis (Fig. 4A, 4B). Importantly, this simulation did not reveal a single K^+ permeation event following TEA binding, in spite of the sustained transmembrane voltage driving K^+ outwards (Fig. 3B); the inhibitor prevents cytoplasmic K^+ ions from reaching S_{cav} (Fig. 4B), and in doing so, it precludes the onset of knock-on mechanism of permeation through the selectivity filter described in the previous section (Fig. 2B).

RY785 binding to Kv2.1 permits K^+ flow and involves residues in the cytoplasmic gate

As mentioned, RY785 is a specific, potent inhibitor of Kv2.1 (IC_{50} = 50 nM) (12), but the structural basis for this inhibitory activity is unknown. Unlike TEA, RY785 is electroneutral, and significantly larger, and thus it might have a very different mode of interaction with the channel. To examine this interaction and its mechanistic implications, we carried out an MD simulation identical in every way to that described above for TEA. (Note this simulation required development and optimization of a set of forcefield parameters for RY785, as described in Methods and in Supplementary Information.) This simulation also showed that RY785 can readily penetrate the cytoplasmic gate, though more slowly than TEA (Fig. 3D). While RY785 is not cationic, it features an electric dipole, and the 100-mV voltage difference applied across the simulation system (positive inside) orients the inhibitor as it enters the channel. Specifically, the m-methoxybenzene moiety passes through the gate first and is ultimately closer to the selectivity filter, while the benzimidazole and thiazole moieties, which are more electronegative, cross the gate last and thus end up being closer to the cytoplasmic space (Fig. 3D, 3F). Like TEA, RY785 continued to be very dynamic inside the pore, though it primarily sampled a unique binding mode, in four different configurations reflecting the symmetry of the channel; displacements parallel to the pore axis were far less common than for TEA, and RY785 did not escape from the protein interior in a 5.5-μs trajectory (Fig. 3E, 3F). Nevertheless, we observed that RY785 did not prevent the flow of K^+ ions through the channel, nor did it significantly alter the knock-on mechanism within the filter (Fig. 3E). Unlike TEA, RY785 does not prevent cytoplasmic K^+ ions from reaching the S_{cav} site, though they clearly do so more rarely; thus the permeation rate we observe is approximately 4 times slower that for the uninhibited channel (Fig. 3E).

Further inspection of the simulation data reveals why RY785 does not block the outward flow of K^+ ions. As shown in Fig. 4, this inhibitor binds to the wall of the cavity, through the benzimidazole and thiazole moieties; the m-methoxybenzene ring projects toward the center of the pore, but the

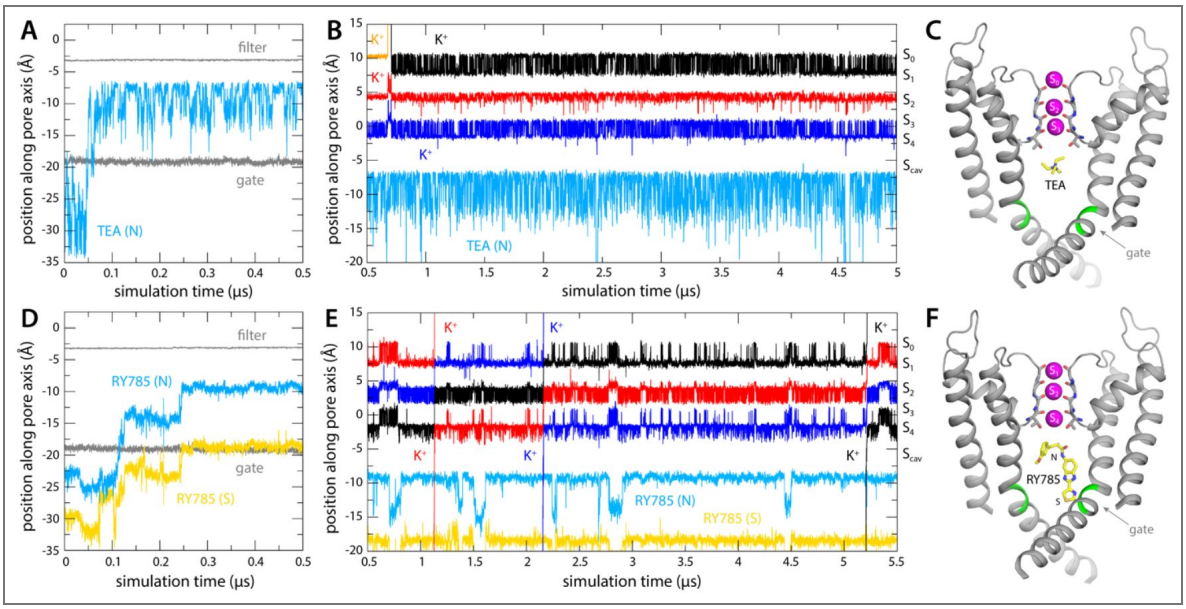


Figure 3. Binding of TEA and RY785 to Kv2.1 channel and impact on K^+ permeability.

(**A**) Time trace of the position of TEA (N atom) in the first 500 ns of the simulation, showing its spontaneous binding to the cavity between the selectivity filter and the cytoplasmic gate (marked by the Cα atoms of T373 and P406, respectively, indicated with gray traces). (**B**) Time trace of the position of TEA for the rest of the 5-μs simulation, alongside those for K^+ ions within the selectivity filter, shown as in Figure 2. The location of each of the K^+ binding sites therein is indicated alongside the plot. (**C**) Snapshot of the final snapshot of the simulation, with TEA bound to S_{cav}. Only two of the four channel subunits are shown, for clarity. Residues P406 and I405 are marked in green. (**D-F**) Same as (A-C), for the simulation of RY785 binding, indicating separately the positions of the central N atom and of the S atom in the distal 5-membered ring.

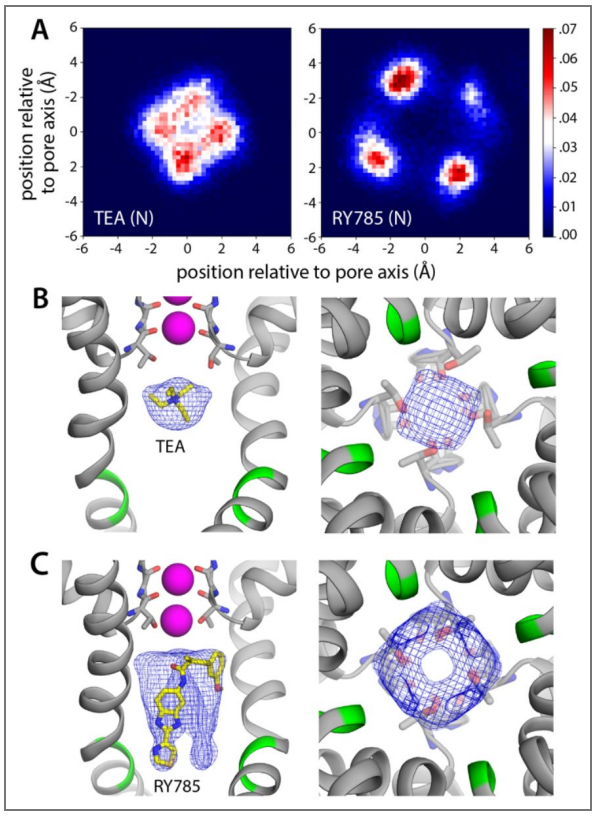


Figure 4. Bound RY785 does not occlude the K^+ pathway, but TEA does.

(**A**) Probability histograms for the position of TEA and RY785 (in Å) as projected on the plane of the membrane, i.e. perpendicular to the pore, with the origin on its central axis. (**B**) Snapshot of the simulation of Kv2.1 and TEA described in Fig. 3, with a density map for TEA (blue mesh) calculated for all non-hydrogen atoms and all simulation snapshots. The map is viewed from the membrane plane, on the left, as well as from the cytoplasmic entrance of the pore, on the right. (**C**) Same as (B), for the simulation of Kv2.1 and RY785. All maps are contoured at the same sigma value (0.04).

high flexibility of this segment leaves an open pathway for K^+ throughout the length of the cavity. Logically, this pathway is narrower than for the apo protein, explaining the slower permeation rate, but it is sufficient to permit K^+ to access the S_{cav} site and ultimately enter the selectivity filter. This particular mode of interaction stems from persistent hydrophobic interactions with residues in the S6 helices (Fig. 5). In particular, the most important contacts are Val409, Pro406, Ile405, Ile401, and Val398, in one or other subunit of the tetramer; among these, Pro406, Ile401, and Val398 appear to be key, as the inhibitor often contacts these residues simultaneously in two protein subunits (Fig. 5A). As will be discussed below, this observation is important, because several of these residues are very likely to form the hydrophobic seal that closes the cytoplasmic entrance to the pore in the deactivated, closed state of the channel.

Bound TEA and RY785 differentially impact K^+ access to the selectivity filter

To corroborate the results described above, we used an alternative simulation design wherein no voltage difference was applied across the membrane to drive K^+ outwards. Instead, we induced a knock-on event within the selectivity by forcibly driving the central K^+ ion (in either S_2 or S_3) to the S_1 site, reasoning this perturbation would result in the immediate ejection of the outermost K^+, the movement of the innermost K^+ into S_2 or S_3, and the creation of an artificial vacancy in the S_4 site. With either TEA or RY785 occupying the water-filled cavity, just as in the simulations described above, we asked which inhibitor would permit or preclude reloading of site S_4 by a K^+ ion entering the pore through the cytoplasmic gate.

As shown in Fig. 6, the ion dynamics within the selectivity filter was as anticipated, reproducibly and irrespective of what inhibitor is bound to the channel. The simulations with TEA and RY785 clearly differ otherwise. While TEA is bound, the S_4 site remains vacant after the induced knock-on event; only when TEA spontaneously diffuses back into the cytoplasm (note no electric field is applied) prior to the knock-on event, do we observe a K^+ ion quickly binding to S_4, thereby restoring the preferred 3-ion configuration (Fig. 6A). Reloading of a K^+ is also what we observed in all the simulations we carried out with bound RY785, consistent with the observation this inhibitor does not physically occlude the pathway for K^+ into the selectivity filter in the open state of the channel (Fig. 6B). These results are completely coherent with those obtained when voltage was used as a driving force for K^+ flow in demonstrating that TEA is an open-channel blocker but RY875 is not.

Discussion

Previous experimental studies have shown that RY785 is a potent, selective inhibitor of Kv2 channels (12). Nonetheless, its site of recognition and precise mechanism of action have been unknown. Allosteric inhibitors featuring thiazole moieties, like RY785 does, have been shown to interact with the voltage-sensing domains of several voltage-gated Na^+ channels (26, 27), suggesting RY785 could act similarly. However, available electrophysiological evidence shows that RY785 competes with TEA, a well-studied K^+-channel inhibitor known to bind within the pore domain (13); it seems very probable therefore that the binding sites for these two inhibitors coincide, at least in part. The computer simulations of Kv2.1 presented in this study were designed to support or refute this notion without a priori assumptions, but ultimately, the results support that interpretation: when the cytoplasmic gate of the channel is open, both RY785 and TEA can enter the K^+ permeation pathway (though not simultaneously), and ultimately dwell in a binding site within the water-filled cavity that precedes the selectivity filter. Aside from this similarity, however, the mechanisms of action of these two inhibitors seem entirely different.

TEA is a small tetrahedral molecule, and is positively charged, though it is larger than a K^+ ion. Thus, under positive-inside voltages, it quickly enters the ion permeation pathway and binds near the entrance of the selectivity filter, which is too narrow for TEA to penetrate. Because the molecular symmetry of TEA does not match the 4-fold symmetry of the channel, TEA tends to dwell slightly off-axis; however, this deflection is smaller than the size of a K^+ ion, and so on

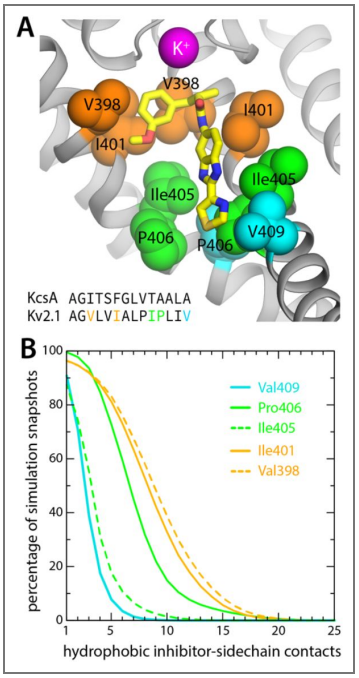


Figure 5. RY785 binds to the interior wall in the Kv2.1 cavity.

(**A**) Close-up of the snapshot shown in Fig. 4C, highlighting the most frequently observed protein contacts for RY785; these are on helix S6, which lines the cavity. Only two adjacent protein subunits are shown, for clarity. Note V409, P406 and Ile405 are at the narrowest point of the permeation pathway (aside from the selectivity filter). For reference, the inset shows an alignment of the sequence of this region of S6 (residues 396 to 409) with its equivalent in the KcsA K^+ channel (residues 98 to 111), whose closed-state structure is known (25). A more comprehensive comparison of the sequences of the S6 segment in K^+ and Ca^{2+} channels is included in Supp. Fig. 1 (**B**) Statistical analysis of the interactions depicted in panel (A). A contact was defined as an instance wherein a C atom in the abovementioned hydrophobic sidechains was within 4.5 Å of one of the C/S atoms in RY785; a given snapshot might thus show multiple contacts with each side-chain, sometimes in two different subunits. The plot in the figure quantifies the percentage of simulation snapshots in which a given number of contacts were observed for each side-chain, at minimum; e.g. in 80% of the snapshots RY785 forms ~5 or more contacts with Pro406; in 1/4 of those, or 20% of the total, the number of observed contacts is ~10 or more. Contacts with Val409 and Ile405 are mostly in one subunit, while Pro406, Ile401 and Val398 are contacted simultaneously in two subunits.

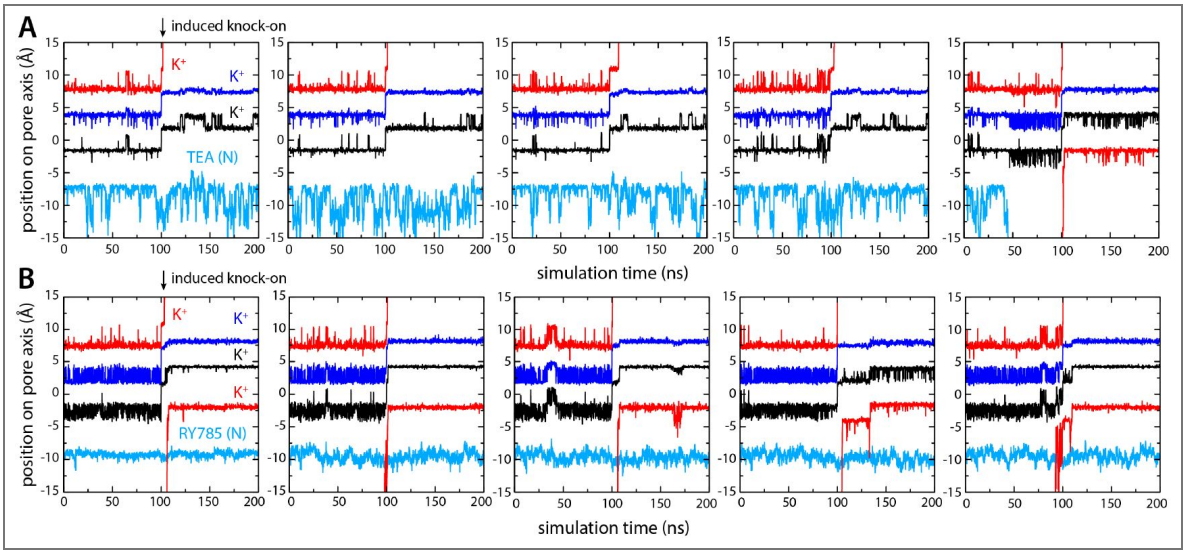


Figure 6. **Bound TEA precludes K^+ from accessing S_4 binding site, but bound RY785 allows it.**

(**A**) Five independent simulations of Kv2.1 bound to TEA wherein a knock-on event was artificially induced in the selectivity filter at t = 100 ns, to create a K^+ vacancy in site S_4. K^+ did not reload this site in the subsequent 100 ns, except when TEA spontaneously dissociated prior to the knock-on event (right). (**B**) Same as (A), for Kv2.1 bound to RY785. In all simulations, a K^+ ion reloads the S_4 site within ~10 ns of the induced knock-on event, corroborating RY785 is not an open-state blocker of Kv2.1.

average the blocker appears to be on the channel axis. Consistent with its location and charge, we observe that TEA completely blocks the outward permeation of K^+ ions under our simulation conditions; in contrast, in an independent simulation under the same conditions, but in the absence of TEA, we observe instead fast K^+ throughput, at a rate comparable to experimental measurements. Comparison of these two simulations shows that TEA blocks K^+ currents because it prevents K^+ ions in the cytoplasmic space from destabilizing the K^+ ions that reside in the selectivity filter.

RY785, however, does not block the flow of K^+ ions through the channel, while the cytoplasmic gate is open. RY785 is electroneutral, and mostly hydrophobic; thus, despite being larger than TEA, RY785 does not tend to occupy the center of the pore. Instead, it packs against the walls of the cavity, forming interactions with a cluster of hydrophobic sidechains that, collectively, is specific to Kv2 channels (Fig. 5, Supp. Fig. 1). Recognition of RY785 therefore does not occlude the pathway for K^+ into the selectivity filter. How does RY785 inhibit Kv2 channels then? A puzzling but revealing experimental observation is that RY785 recognition by Kv2.1 channels, following activation, accelerates their deactivation (13). That is, RY785 appears to trap itself within the cavity, upon closure of the extracellular gate. Under normal conditions, gate closure requires the repolarization of the membrane, i.e. an energy input. Negative-inside voltages drive the S4 helix in each of the voltage sensors to the down state (creating the so-called gating currents), and this motion is communicated to the pore domain via the S4-S5 linker, ultimately causing a rearrangement in helices S5 and S6, which closes the cytoplasmic gate (Fig. 7). Clearly, from its binding site in the interior of the pore domain, RY785 cannot directly impact the voltage sensors, nor their mechanical coupling with S5-S6. Nonetheless, our simulations indicate an indirect effect. We observe that RY785 forms persistent interactions with the hydrophobic residues that are likely to constrict the pore in the closed state, in the S6 helix (17); in several cases, RY785 interacts simultaneously with the same residue in two adjacent channel subunits. These observations suggest that RY785 might stabilize a semi-closed conformation of the gate that is no longer permeable to K^+, even though the structural rearrangement in S5-S6 that is required for full closing has not been achieved completely (Fig. 7). That is, an opening between the four protein subunits would remain at the position of the gate, but this opening would be occupied by RY785, bridging hydrophobic interactions between protein residues therein. Importantly, this hypothetical conformation might not require the voltage sensors to reach the down state completely (Fig. 7); if so, K^+ currents would be terminated more readily upon repolarization with bound RY785 than otherwise. This model would also explain the experimental observation that RY785 partially inhibits the currents generated by the motion of the S4 helix, known as gating currents (13), even though S4 is about 3 nm away from the RY785 binding site.

While further studies will be required to fully validate or refute this mechanistic model, recently published work supports our hypothesis. After this study was first reported in 2024, the structure of a Kv2.1 channel with a closed pore domain was successfully determined (29). That structure shows that a Pro-Ile-Pro motif in helix S6 marks the position of the intracellular gate, where the pore domain constricts maximally (aside from the selectivity filter). As illustrated in Fig. 5, this motif is precisely where the benzimidazole and thiazole moieties of RY785 are recognized in our simulations. The inhibition mechanism we outline in Fig. 7 thus seems increasingly plausible. Nonetheless, the fact that both open and closed states of the pore are now known provides an excellent opportunity to examine this model further. Specifically, it is now possible to map the intrinsic conformational free-energy landscape of the pore domain, in a simplified construct lacking the voltage sensors, using enhanced-sampling simulations (30); we anticipate that both open and closed states of the gate will be identifiable free-energy minima in this landscape, and that the closed state will be less favorable than the open state (in absence of the voltage sensors). Comparison of analogous calculations carried out with and without bound RY785, alongside an evaluation of the changes in K^+ permeability in each case (31), would provide further quantitative insights into the intriguing mechanism by which RY785 inhibits gating and ion flow in Kv2 channels, and in turn, aid future optimizations of this inhibitor.

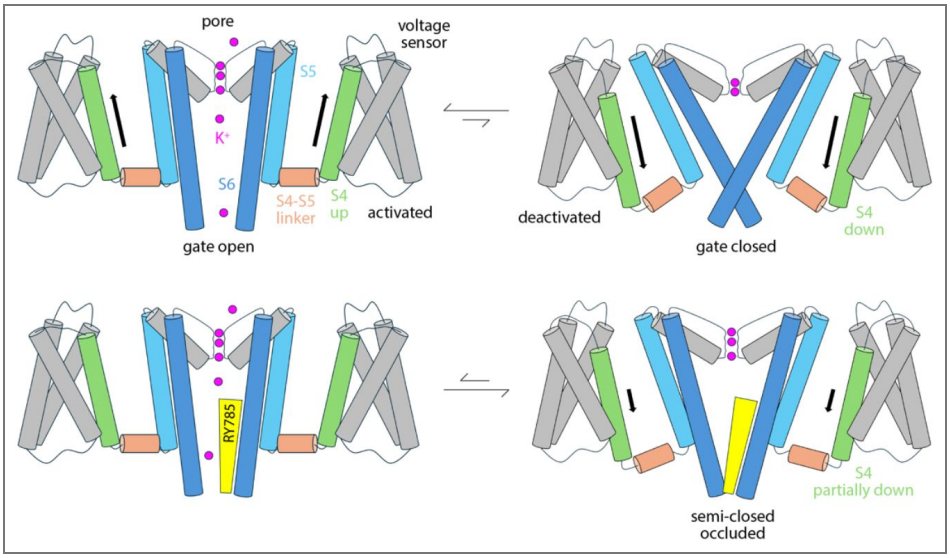


Figure 7. Hypothetical mode of Kv2-channel inhibition by RY785.

R785 does not directly block K^+ flow; instead, it reshapes the conformational free-energy landscape of the pore domain to stabilize an occluded, partially closed state that does not require S4 to reach the down state in full. The model is an inference based on the simulation data reported in this article and a previous electrophysiological study by Sack and co-workers (13). Note this is a simplified diagram of the functional cycle of the channel, which includes non-conductive or transiently inactivated states, aside from the fully deactivated form (19, 28).

Methods

Molecular Dynamics simulations of K^+ flux through Kv2.1 in 100 mV

The MD simulations of activated wild-type Kv2.1 are based on the cryo-EM structure of the activated, open state (PDB entry 8SD3) (28). In all cases, the simulations examined a construct encompassing residues 174 to 426 (S1 to S6), with neutral N- and C-termini and all ionizable side-chains in their default protonation state at pH 7 (the net charge of these constructs is thus zero). Two K^+ ions were initially positioned in the selectivity filter, one coordinated by residues 373 and 374 and another by residues 375 and 376; a third ion was positioned below the side chain of 373, outside the filter, and two water molecules were modeled between the three ions. (This configuration was very short lived and replaced by others where 3 K^+ ions concurrently occupy the filter, typically no water molecules in between – see Results.) For all systems we used Dowser (32) to model structural water molecules within the protein that were not resolved in the experimental density maps. To complete the initial simulation setup of the experimental cryo-EM structure, the construct including ions and water molecules was energy-minimized using CHARMM (33) and the CHARMM36m force field (34-36); specifically, the minimization used the steepest-descent algorithm, for 250 steps, and then conjugate-gradient algorithm, for another 250 steps.

The channel was simulated in a POPC lipid bilayer and a 300 mM KCl solution. (Note that while this concentration is atypical in the intracellular environment, it is often used in electrophysiological studies. In the simulation context, this choice is sensible in that it leads to a greater number of permeation events, and therefore, greater statistical confidence in the calculated ion conductance, or its inhibition.) To generate a molecular model of this membrane/solvent environment, we created a coarse-grained (CG) POPC-lipid bilayer in 300 mM KCl in an orthorhombic box of ~150 × 150 × 100 Å using insane.py (37). To equilibrate this CG system, we carried out a 20-μs MD simulation using GROMACS 2018.8 (38) and the MARTINI 2.2 force field (39, 40) at constant temperature (303 K) and constant semi-isotropic pressure (1 atm) and with periodic boundary conditions. The integration time-step was 20 fs. We then embedded the structure of the Kv2.1 channel in this environment; to do so, the atomic structure of the channel was first coarse-grained with martinize.py (39), and overlaid onto the equilibrated membrane/solvent system, removing all overlapping lipid and water molecules. Then, to optimize the resulting protein/lipid/solvent interfaces, we carried out a 10-μs MD simulation of the complete system using GROMACS 2018.8 (38) and MARTINI 2.2 (39, 40) at constant temperature (303 K) and constant semi-isotropic pressure (1 atm) and with periodic boundary conditions and an integration time step of 20 fs. Having ascertained the equilibration of the membrane structure and of the position and orientation of the protein in the lipid bilayer, the final snapshot of the CG trajectory was transformed into an all-atom representation compatible with the all-atom CHARMM36m force field (34-36). To do so, lipid and solvent molecules were back-mapped onto all-atom models (41), while the CG version of Kv2.1 was replaced (not back-mapped) with the energy-minimized all-atom construct described above, after optimally superposing the Cα trace of the latter onto that of the former. The resulting all-atom molecular system includes 526 POPC lipids, 38,629 water molecules, and 208 K^+ and 239 Cl^- (300 mM KCl) in an orthorhombic box of ca. 150 × 150 × 100 Å. To further optimize this all-atom model, the simulation system was first energy-minimized for 5,000 steps with NAMD 2.13 (42, 43) and CHARMM36m (34-36), using the conjugate-gradient algorithm. We then carried out a series of MD simulations wherein structural restraints applied to the protein and ions/water within the selectivity filter are progressively weakened over the course of ~150 ns, as previously described (19, 28). These simulations were carried out using NAMD 2.13 (42, 43) and CHARMM36m (34-36) at constant temperature (298 K) and constant semi-isotropic pressure (1 atm) with periodic boundary conditions and an integration time-step of 2 fs. Electrostatic interactions were calculated using with the Particle-Mesh Ewald method (44), with a real-space cutoff value of 12 Å; van der Waals interactions were also cut off at 12 Å, with a smooth switching function taking effect at 10 Å.

To quantify the ion-conducting properties of activated Kv2.1, a 25-μs MD trajectory was calculated under an applied voltage (positive inside) using an ANTON2 supercomputer (45) and the all-atom CHARMM36m force field (34-36). The starting configuration for this simulation was the final configuration of the abovementioned equilibration process. The simulation was again carried out at constant temperature (298 K) and semi-isotropic pressure (1 atm), in this case set with the Nose-Hoover thermostat (46, 47) and the Martyna-Tobias-Klein barostat (48), respectively, and with periodic boundary conditions and an integration time-step of 2.5 fs. Electrostatic interactions were calculated using the Gaussian-split Ewald method (49); van der Waals interactions were cut off at 10 Å. To preclude large-scale changes in the secondary or tertiary structure of the channel that might develop in the 25-μs timescale, due to cumulative forcefield inaccuracies, the energy function of the simulation was supplemented with a weak biasing potential that favors the known experimental geometry, while permitting the structure to locally fluctuate as required for ligand recognition and ion permeation (19, 28). This potential acts on all Φ, $\psi$and χ_1 dihedral angles in the protein, and is defined by:

$$U(\theta_t) = k\sum_{m=1}^{m=6}(-1)^m\left[1+\cos\left(m\theta_t - m\left(\theta_{expt}-180\right)\right]\right/ m!$$

where θ_t is the value of each dihedral angle at time t in the simulation, θ_{expt} denotes the corresponding value in the experimental structure, and $k = 1\ k_BT$. Note this potential is identical to that used in previous studies based on multi-microsecond ANTON2 simulations (50-52), except the magnitude of the bias in this study is considerably weaker (i.e. smaller k). To drive K^+ ions outward, a transmembrane voltage difference was applied across the membrane, positive inside. This voltage difference was applied as a jump from 0 to 100 mV at timepoint 0.5 μs and sustained thereafter. The desired voltage resulted from application of an outwardly directed, constant electric field perpendicularly to the membrane plane (53). For a simulation box of approximately 92 Å in that direction, a voltage difference of 100 mV corresponds to an electric field of 0.024 kcal mol^{-1} $Å^{-1}$ e^{-1} (note 1 kcal mol^{-1} $Å^{-1}e^{-1}$ = 43.4 mV/Å).

Simulations TEA and RY785 binding and inhibition in 100 mV

Additional MD simulations were carried out to examine the mode of TEA and RY785 binding and inhibition. These simulations used as starting point the final configuration of the 25-μs MD trajectory of described above; a molecule of TEA or RY785 was added about 10 Å below the cytoplasmic gate, and overlapping water molecules were removed. These constructs were then energy-minimized using CHARMM (33) and the CHARMM36m force field (34-36); specifically, the minimization consisted of 100 steps using the steepest-descent algorithm with the protein and K^+ ions within fixed. To further optimize these all-atom models, the simulation systems were then energy-minimized for 5,000 steps with NAMD 2.13 (42, 43) and the CHARMM36m force field (34-36), using the conjugate-gradient algorithm. Again using NAMD 2.13, we then carried out a 20-ns MD simulation of each channel-inhibitor system wherein weak structural restraints identical to those used in the 25-μs MD trajectory were applied to the protein. In addition, a flat-bottom confining potential was applied to TEA or RY785 to keep the inhibitor inside a cylindrical volume co-axial with the channel pore, 20 Å in diameter. Specifically, we used atoms Y376:C_β and V409:C_β in the four protein subunits to define this axis, and the inhibitor was confined by a potential of the form:

$$U(d_t) = 0.5\ k\ (d_t - d_o)^2\ \ if\ d_t > d_o$$
$$U(d_t) = 0\ \ if\ d_t \le d_o$$

where d_t is the distance between the center-of-mass of the inhibitor and the channel axis, perpendicularly to that axis, d_o is 10 Å and k = 100 kcal/mol/$Å^2$. To preclude the inhibitor from diffusing across the periodic boundary of the system (i.e. preserving it in the cytoplasmic side of the channel), a confining potential of the same functional form was also applied relative to the center of the four V409:C_β atoms; in this case d_t is the projection of the distance onto the channel

axis, while d_o is 15 Å. Following these 20-ns equilibrations, trajectories of 5-μs and 5.5-μs were calculated on ANTON2 for the TEA and RY785 systems respectively, using exactly the same settings as those reported above for the channel-only simulation.

Simulations TEA and RY785 inhibition after induced K^+ knock-on event

The simulations described in Fig. 6 used as starting point the final configurations of the trajectories calculated with ANTON2 for the channel-inhibitor systems (Fig. 3). In these starting configurations, three K^+ ions occupy the selectivity filter, and TEA or RY785 resides within the cavity. To create a vacancy in the S_4 site, without a voltage difference across the membrane, the central K^+ ion (which fluctuates between S_2 and S_3) was driven outwards at t = 100 ns, by activating a set of restraints that bring the ion within coordination distance of the carbonyl O atoms of Y376 and G375 in all four subunits (ca. 3 Å, k = 3 kcal/mol/Å^2). As a result, the outermost K^+ ion (which fluctuates between S_0 and S_1) was ejected, and the innermost K+ ion moved to the center of the filter, creating the desired vacancy in S_4. The simulations were then continued for 100 ns to examine whether or not K^+ ions would reload this vacancy. These simulations were carried out using NAMD 3.0b6 (42, 43) and the CHARMM36m force field (34-36). All other settings, including confining potentials, were as described above.

Development of molecular-mechanics forcefield for RY785

To our knowledge, no molecular-mechanics (MM) forcefield for RY785 was publicly available prior to this work. Thus, a new forcefield had to be developed and optimized to be able to introduce this molecule in our simulations of Kv2.1 – specifically one compatible with the CHARMM General Force Field, or CGenFF (54). Following recommended procedures (54-56), we carried out a series of quantum chemical calculations to generate ab-initio benchmarking data, including atomic charges, optimized molecular geometries and multiple potential energy surfaces (PES) generated by relaxed-scan of selected bond- and dihedral-angles; these calculations were performed in the gas phase using Gaussian 16 (Gaussian, Inc., Wallingford CT, 2016), using the MP2/6-31G(d) method. Structural properties and calculated PESs were then used to adjust atomic-charge, bond-angle and dihedral-angle parameters lacking adequate entries in CGenFF. These MM parameters were refined targeting the benchmark QM data, including interaction energies and H-bond distances for multiple RY785-H_2O binary complexes. Each of these RY785-H_2O conformers probes the interaction of a H_2O molecule with a different H-bond donor (C-H, or N-H) or acceptor (N or S) in RY785. The complexes were set up with an ideal H-bond interaction between RY785, in its MP2/6-31G(d) optimized geometry, and a water molecule, in its idealized TIP3P geometry (57). The interaction distances in these complexes were optimized at the HF/6-31G(d) level while keeping all other degrees of freedom fixed; the interaction energy was then calculated without correction for basis set superposition error and multiplied by a factor 1.16, as suggested elsewhere (56). Detailed results are provided in Supplementary Information.

Data availability

Forcefield parameter files for RY785 can be downloaded from *https://github.com/Faraldo-Gomez-Lab-at-NIH/Download*.

Acknowledgements

This research was funded by the intramural program of the National Heart, Lung and Blood Institute, National Institutes of Health (NIH). Computational resources were provided by the NIH High-Performance Computing Facility 'Biowulf' and by the Pittsburgh Supercomputing Center, which provided access to an Anton2 computer donated by D.E. Shaw Research and supported through NIH grant R01GM116961.

Additional information

Author contributions

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Conceptualized research: José D. Faraldo-Gómez

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Funding

Funder	Grant reference number	Author
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Additional files

Supplemental Information

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Peer reviews

Reviewer #1 (Public review):

Summary:

The authors were seeking to identify a molecular mechanism whereby the small molecule RY785 selectively inhibits Kv2.1 channels. Specifically, the authors sought to explain some of the functional differences that RY785 exhibits in experimental electrophysiology experiments as compared to other Kv inhibitors, namely the charged and non-specific inhibitor tetraethylammonium (TEA). The authors used a recently published cryo-EM Kv2.1 channel structure in the open activated state and performed a series of multi-microsecond-long all-atom molecular dynamics simulations to study Kv2.1 channel conduction under the applied membrane voltage with and without RY785 or TEA present. They observed that while TEA directly blocks K+ permeation by occluding ion permeation pathway, RY785 binds to multiple non-polar residues near the hydrophobic gate of the channel driving it to a semi-closed non-conductive state. They confirmed this mechanism using an additional set of simulations and used it to explain experimental electrophysiology data,

Strengths:

The total length of simulation time is impressive, totaling many tens of microseconds. The authors develop their own forcefield parameters for the RY785 molecule based on extensive QM based parameterization. The computed permeation rate of K+ ions through the channel observed under applied voltage conditions is in reasonable agreement with experimental estimates of the single channel conductance. The authors have performed extensive simulations with the apo channel as well as both TEA and RY785. The simulations with TEA reasonably demonstrate that TEA directly blocks K+ permeation by binding in the center of the Kv2.1 channel cavity, preventing K+ ions from reaching the SCav site. The authors conclude that RY785 likely stabilizes a partially closed conformation of the Kv2.1 channel and thereby inhibits K+ current. This conclusion is plausible given that RY785 makes stable contacts with multiple hydrophobic residues in the S6 helix, which they can also validate using a recently published closed-state Kv2.1 channel cryo-EM structure. This further provides a possible mechanism for the experimental observations that RY785 speeds up the deactivation kinetics of Kv2 channels from a previous experimental electrophysiology study.

Weaknesses:

The authors, however, did not directly observe this semi-closed channel conformation and in fact acknowledge that more direct simulation evidence would require extensive enhanced-sampling simulations beyond the scope of this study. They have not estimated the effect of RY785 binding on the protein-based hydrophobic pore constriction, which may further substantiate their proposed mechanism. And while the authors quantified K+ permeation, they have not made any estimates of the ligand binding affinities or rates, which could have been potentially compared to experiment and used to validate their models.

However, despite those relatively minor weaknesses, the conclusions of the study are convincing, and overall this is a solid study helping us to understand two distinct molecular mechanisms of the voltage-gated potassium channel Kv2.1 inhibition by TEA and RY785, respectively.

https://doi.org/10.7554/eLife.101855.2.sa2

Reviewer #2 (Public review):

Summary

In this manuscript, Zhang et al. investigate the conduction and inhibition mechanisms of the Kv2.1 channel, with a particular focus on the distinct effects of TEA and RY785 on Kv2 potassium channels. Using microsecond-scale molecular dynamics simulations, the authors characterize K^+ ion permeation and RY785-mediated inhibition within the central pore. Their results reveal an inhibition mechanism that differs from those described for other Kv channel inhibitors.

Strengths

The study identifies a distinctive inhibitory mode for RY785, which binds along the channel walls in the open-state structure while still permitting a reduced level of K^+ conduction. In addition, the authors propose a long-range allosteric coupling between RY785 binding in the central pore and changes in the structural dynamics of Kv2.1. Overall, this is a well-organized and carefully executed study, employing robust simulation and analysis methodologies. The work provides novel mechanistic insights into voltage-gated potassium channel inhibition and may offer useful guidance for future structure-based drug design efforts.

Weaknesses:

The study needs to consider the possibility of multiple binding sites for PY785, particularly given its impact on voltage sensors and gating currents. Specifically, the potential for allosteric binding sites in the voltage-sensing domain (VSD) should be assessed, as some allosteric modulators with thiazole moieties are known to bind VSD domains in multiple voltage-gated sodium channels (Ahuja et al., 2015; Li et al., 2022; McCormack et al., 2013; Mulcahy et al., 2019). Increasing structural and functional evidence supports the existence of multiple ligand-binding modes in voltage-gated ion channels. For example, polyunsaturated fatty acids have been shown to bind to KCNQ1 at both the voltage sensor domain and the pore domain (*https://doi.org/10.1085/jgp.202012850*). Similarly, cannabidiol has been structurally resolved in Nav1.7 at two distinct sites, one in a fenestration and another near the IFM-binding pocket (*https://doi.org/10.1038/s41467-023-39307-6*). These advances illustrate that ligand effects cannot always be interpreted based solely on a single binding site identified previously.

https://doi.org/10.7554/eLife.101855.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***eLife Assessment***
>
> *This study represents an important advance in our understanding of how certain inhibitors affect the behavior of voltage gated potassium channels. Robust molecular dynamics simulation and analysis methods lead to a new proposed inhibition mechanism with strength of support being mostly convincing, and incomplete in some*

> *aspects. This study has considerable significance for the fields of ion channel physiology and pharmacology and could aid in development of selective inhibitors for protein targets*

We are encouraged by this favorable assessment and thank editors and reviewers for their constructive feedback and recommendations. We trust that the revisions made to the manuscript will clarify the aspects that had been perceived to be incomplete.

> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *This study seeks to identify a molecular mechanism whereby the small molecule RY785 selectively inhibits Kv2.1 channels. Specifically, it sought to explain some of the functional differences that RY785 exhibits in experimental electrophysiology experiments as compared to other Kv inhibitors, namely the charged and non-specific inhibitor tetraethylammonium (TEA). This study used a recently published cryo-EM Kv2.1 channel structure in the open activated state and performed a series of multi-microsecond-long all-atom molecular dynamics simulations to study Kv2.1 channel conduction under the applied membrane voltage with and without RY785 or TEA present. While TEA directly blocks K+ permeation by occluding ion permeation pathway, RY785 binds to multiple nonpolar residues near the hydrophobic gate of the channel driving it to a semi-closed non-conductive state. This mechanism was confirmed using an additional set of simulations and used to explain experimental electrophysiology data.*
>
> *Strengths:*
>
> *The total length of simulation time is impressive, totaling many tens of microseconds. The study develops forcefield parameters for the RY785 molecule based on extensive QM-based parameterization. The computed permeation rate of K+ ions through the channel observed under applied voltage conditions is in reasonable agreement with experimental estimates of the singlechannel conductance. The study performed extensive simulations with the apo channel as well as both TEA and RY785. The simulations with TEA reasonably demonstrate that TEA directly blocks K+ permeation by binding in the center of the Kv2.1 channel cavity, preventing K+ ions from reaching the SCav site. The conclusion is that RY785 likely stabilizes a partially closed conformation of the Kv2.1 channel and thereby inhibits the K+ current. This conclusion is plausible given that RY785 makes stable contact with multiple hydrophobic residues in the S6 helix. This further provides a possible mechanism for the experimental observations that RY785 speeds up the deactivation kinetics of Kv2 channels from a previous experimental electrophysiology study.*
>
> *Weaknesses:*
>
> *The study, however, did not produce this semi-closed channel conformation and acknowledges that more direct simulation evidence would require extensive enhanced-sampling simulations. The study has not estimated the effect of RY785 binding on the protein-based hydrophobic pore constriction, which may further substantiate their proposed mechanism. And while the study quantified K+ permeation, it does not make any estimates of the ligand binding affinities or rates, which could have been potentially compared to the experiment and used to validate the models.*

As stated in the original manuscript, we concur that the mechanism we propose remains hypothetical until further studies of the complete conformational cycle of the channel are conducted. The recently determined structure of a Kv2.1 channel in the closed state (Mandala and MacKinnon, PNAS 2025) presents an excellent opportunity to do so. Indeed, a cursory analysis of that structure shows that a Pro-Ile-Pro motif in helix S6 marks the position of the

intracellular gate, where the pore domain constricts maximally (aside from the selectivity filter). As illustrated in Fig. 5, this motif is precisely where the benzimidazole and thiazole moieties of RY785 bind in our simulations. The mechanism we outline in Fig. 7 thus seems very plausible, in our view; that is RY785 occludes the K^+ permeation pathway before the pore domain reaches the closed conformation, explaining the observed electrophysiological effects (see Discussion). The Discussion has been revised to note the recent discovery of the aforementioned structure, its implications for the mechanism we propose, and the opportunities for further research that are now open.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *In this manuscript, Zhang et al. investigate the conductivity and inhibition mechanisms of the Kv2.1 channel, focusing on the distinct effects of TEA and RY785 on Kv2 potassium channels. The study employs microsecond-scale molecular dynamics simulations to characterize K+ ion permeation and compound binding inhibition in the central pore.*
>
> *Strengths:*
>
> *The findings reveal a unique inhibition mechanism for RY785, which binds to the channel walls in the open structure while allowing reduced K+ flow. The study also proposes a long-range allosteric coupling between RY785 binding in the central pore and its effects on voltage-sensing domain dynamics. Overall, this well-organized paper presents a high-quality study with robust simulation and analysis methods, offering novel insights into voltage-gated ion channel inhibition that could prove valuable for future drug design efforts.*
>
> *Weaknesses:*
>
> *(1) The study neglects to consider the possibility of multiple binding sites for RY785, particularly given its impact on voltage sensors and gating currents. Specifically, there is potential for allosteric binding sites in the voltage-sensing domain (VSD), as some allosteric modulators with thiazole moieties are known to bind VSD domains in multiple voltage-gated sodium channels (Ahuja et al., 2015; Li et al., 2022; McCormack et al., 2013; Mulcahy et al., 2019).*

As noted in the manuscript, we designed our simulations to explore the possibility that RY785 binds within the pore domain, because TEA and RY785 are competitive and TEA is known to bind within the pore. That RY785 did in fact spontaneously and reproducibly bind within the pore was however not a predetermined outcome; if the site of interaction for the inhibitor was elsewhere in the channel, the simulation would not have shown a stable associated state, which would have prompted us to examine other possible sites, including the voltage sensors. It was also not predetermined or foreseeable a priori that the mode of interaction we observed in simulation provides a straightforward rationale for the electrophysiological effects of RY785. Based on our results, therefore, we believe that RY785 binds within the pore of Kv2. As stated by the reviewer, other allosteric modulators are known to bind instead to the sensors; to our knowledge, however, there is no precedent of a small-molecule inhibitor that simultaneously acts on the sensors and the pore domain. We therefore believe that future studies should focus on corroborating or refuting the mechanism we propose, through additional experimental and computational work; if, contrary to our claim, RY785 is found not to bind to the pore domain, it would be logical to explore other possible sites of interaction, as the reviewer suggests. The Discussion has been modified to address this point.

> *(2) The study describes RY785 as a selective inhibitor of Kv2 channels and characterizes its binding residues through MD simulations. However, it is not clear whether the identified RY785-binding residues are indeed unique to Kv2 channels.*

To clarify this question, we have included a multiple sequence alignment as Supplementary Figure 1; the revised manuscript refers to this figure in the Discussion section. The alignment reveals that the cluster of residues forming contacts with RY785 (Val409, Pro406, Ile405, Ile401, and Val398) is indeed specific to Kv2.1. Among Kv channels, Kv3.1 and Kv4.1 exhibit the greatest similarity to Kv2.1 at these positions, but they differ in a crucial substitution: Ile405 in Kv2.1 is replaced by Val. This replacement shortens the sidechain, undoubtedly reducing the magnitude of the hydrophobic interaction between inhibitor and channel (Val is approximately 6 kcal/mol, i.e. 1,000 times, more hydrophilic than Ile). Kv5.1 differs from Kv2.1 at two positions: Pro406 is replaced by His, and Val409 by Ile. The introduction of His abolishes the hydrophobic interaction at that position, and the need for hydration likely perturbs all adjacent contacts with RY785. Lastly, Kv6-Kv10 and Cav channels feature entirely different residues at these positions. Consistent with these findings, a recent study by the Sack lab (*https://elifesciences.org/articles/99410*) has demonstrated that Kv5, Kv6, Kv8, and Kv9 pore subunits confer resistance to RY785, while a high-throughput electrophysiological study carried out by Merck (Herrington et al., 2011) reported that RY785 shows no significant activity against Cav channels. The sequence alignment offers a simple interpretation for these experimental observations, namely that RY785 is recognized by Kv2 channels through the abovementioned hydrophobic cluster within the pore domain.

> *(3) The study does not clarify the details, rationale, and ramifications of a biasing potential to dihedral angles.*

We refer the reviewer to published work, for example Stix et al, 2023 and Tan et al, 2022. We provide additional comments below.

> *(4) The observation that the Kv2.1 central pore remains partially permeable to K+ ions when RY785 is bound is intriguing, yet it was not revealed whether polar groups of RY785 always interact with K+ ions.*

We detected no persistent specific interactions between RY785 and the permeant K+ ions.

> ***Recommendations for the authors:***
>
> ***Reviewer #2 (Recommendations for the authors):***
>
> *The manuscript describes atomistic molecular dynamics (MD) simulations of a voltage-gated potassium channel Kv2.1 using its cryo-EM structure in the open activated state and its inhibition by a classical non-specific cationic blocker tetraethylammonium (TEA) as well as a novel selective inhibitor RY785. Using multi-microsecond-long all-atom MD runs under the applied membrane voltage of 100 mV the authors were able to confirm that the channel structure represents an open conducting state with the computed single-channel conductance lower than experimental values, but still in the same order of magnitude range. They also determined that both TEA and RY785 bind in the channel pore between the cytoplasmic hydrophobic gate and narrow selectivity filter (SF) region near the extracellular side. However, while TEA directly blocks a knock-on K+ conduction by physically obstructing ion access to the SF, the mechanism of action of RY785 is different. It does not directly prevent K+ access to the SF but rather binds to multiple residues in the hydrophobic gate region, which effectively narrows a pore and drives the channel toward a semi-closed nonconductive conformation, which might be distinct from one with the deactivated voltage sensors and closed pore observed at hyperpolarized membrane potentials. However, additional studies beyond the scope of this work might be needed to fully establish this mechanism as suggested by the authors.*

> *The manuscript is written very well and represents a significant advance in the field of ion channel research. I do not have any major issues, which need to be addressed. However, I have several suggestions.*
>
> *For the apo-channel K+ conduction MD simulation under the applied voltage, the authors seem to observe mostly a direct or Coulomb knock-on mechanism across the SF with almost no water copermeation. This is in line with computational electrophysiology studies with dual membrane setup by B. de Groot and others but in disagreement with multiple previous studies by B. Roux and others also using applied electric field and CHARMM force fields as in the present study. I wonder why the outcomes are so different. Is it related to the Kv2.1 channel itself, a relatively small applied electric field used (corresponding to a membrane potential of 100 mV vs. 500-750 mV used in many previous simulations), ion force field (e.g., LJ parameters), or some other factors? Could weak dihedral restraints on the protein backbone and side chains contribute to this mechanism? I also wonder if the authors might have considered different initial SF ion configurations. Related to that, I wonder if the authors observed any SF distortions in their simulations including frequently observed backbone carbonyl flipping and/or dilation/contraction.*

We are aware of these discrepancies between published simulation studies, but cannot offer a satisfactory explanation, beyond speculation. The reviewer is correct that the mechanism of ion permeation we observe is comparable to that reported by de Groot, as we noted in Tan et al, 2022 and Stix et al, 2023. Neither in this nor in those previous studies did we observe any persistent distortions of the selectivity filter – but that outcome was expected by construction. The weak biasing potentials acting on the mainchain dihedral angles allow for local fluctuations but not a persistent deformation, relative to the conductive form determined experimentally.

> *For MD simulations with the ligand present, I wonder if the authors can comment on the effect of the ligand especially RY785 on the pore size or more importantly size of the hydrophobic gate. The presence of the ligand itself would definitely result in a narrower pore, but I also wonder if this would also lead to a rearrangement of pore sidechain and/or backbone residues, which would lead to a narrower pore from a protein itself thus confirming the proposed mechanism of driving the channel towards a semi-closed state. It is easy to compute but I wonder if the presence of weak dihedral restraints may preclude this analysis.*

Yes, while the simulation design used in this study allows for local fluctuations in the mainchain structure and nearly unrestricted sidechain dynamics, changes in either the secondary or tertiary structure of the channel are strongly disfavored. This approach is thus sufficient to examine ligand binding or ion flow in the microsecond timescale but not channel gating. In the revised version of the Discussion, we outline a roadmap for future computational studies of that gating process, on the basis of the open-channel structure we used and the recently determined structure of the closed state.

> *The authors state that RY785 does not block K+ ion, but it does significantly slow the rate of K+ ion access to the pore Scav site. Is this not a part of the mechanism for inhibition of the channel? The authors seem to focus on the primary mechanism of inhibition as the RY785 promoting channel closing, but would it not also reduce K+ current in the open state by slowing the rate of K+ entry into the cavity and selectivity filter? The authors should address this point in the text. I am also somewhat confused that in the MD simulations performed by the authors, there is still some K+ conduction with RY785 in the pore, which is not in 100% agreement with electrophysiology experiments. Does it mean that the channel in the simulations has not yet reached that semiclosed state or a reduced K+ conduction is not observed experimentally?*

The salient experimental observation is RY785 abrogates K+ currents through Kv2 channels (Herrington et al, 2011; Marquis et al, 2022). In our view, that observation can be explained in one of two ways: either RY785 completely blocks the flow of K+ ions across the channel while the pore domain remains in the conductive, open state – like TEA does – or RY785 induces or facilitates the closing of the channel, thereby abrogating K+ flow. The fact that we observe K+ flow while RY785 is bound to the channel is therefore not in disagreement with the electrophysiological measurements, but it does rule out the first of those two possible interpretations of the existing experiments. As it happens, the second possible explanation, i.e. that RY785 facilitates the closing of the pore domain, also provides a rationale for another puzzling experimental observation, namely that RY785 shifts the voltage dependence of the currents produced by the voltage sensors as they reconfigure to open or close the intracellular gate.

> *Also, I wonder if the authors considered that since there are 4 potential equivalent sites in the pore (although, overlapping) more than one RY785 might be needed to prevent K+ conduction, even though the experimental Hill coefficient of ~1 does not indicate cooperativity.*

Admittedly, our simulation design was based on the premise that only one RY785 molecule might be recognized within the pore. Based on the outcome of the simulations, we are confident that this assumption was valid, as the binding pose that we identified rules out multiple occupancy – which would be indeed consistent with a Hill coefficient of ~1.

> *I also wonder if the authors considered estimating ligand binding affinities and/or "on" rates from their simulations to have a more direct comparison with experiments and test the accuracy of their models. There are multiple enhanced sampling techniques allowing to do that, although it can be a study on its own.*

We thank the reviewer for this suggestion, which we will consider for future studies.

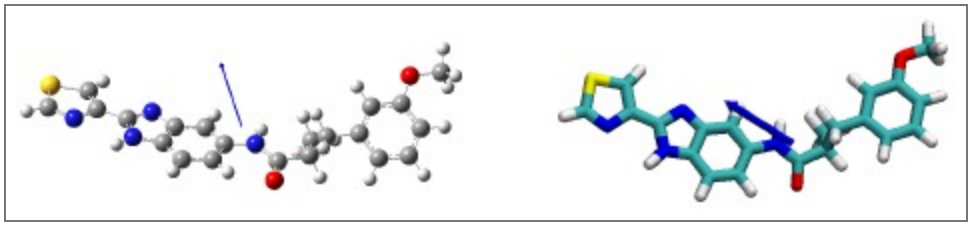
> *The authors also discussed that they could not study Kv2.1 deactivation in a reasonable simulation time. Indeed it is very challenging but they should cite previous studies e.g. 2012 Jensen et al paper (PMID: 22499946) on this subject. There are structures of Kv channels with the deactivated voltagesensing domains (VSDs) available, e..g of EAG1 channel (PDB 8EP1), although they do not have a domain-swapped architecture. There are structural modeling approaches including AlphaFold, which can be potentially used to get a Kv2.1 structure with deactivated VSDs, and targeted MD, string method etc. can be used to study transition between different states with and without bound ligands.*

As noted, a structure of a Kv2 channel with a closed pore has now been determined experimentally. In the revised Discussion, we comment on what this structure tells us about the mechanism of inhibition we propose, and how it could be leveraged in future studies.

> *The authors should be commended for doing a thorough QM-based force field parameterization of RY785. However, a validation of the developed force field*

> *parameters is lacking. In terms of QM validation, a gas-phase dipole moment can be compared in terms of direction and magnitude (it's normal to be overestimated to implicitly reflect solvent-induced polarization). If there are any experimental data available for this compound, they can be tested as well.*

We agree with the reviewer that forcefield validation is important, but to our knowledge no experimental data exists for RY785 to compare with, such as hydration free energies. We did however compare the gas-phase dipole moment computed with QM and with the MM forcefield we developed based on atomic charges optimized to reproduce QM interactions with water. The MM model yields a gas-phase dipole moment of 3.94 D, which is 20% greater than the QM dipole moment, or 3.23 D. That deviation is within the typical range for electroneutral molecules (Vanommeslaeghe et al, 2010), and as the reviewer notes, reflects the solvent-induced polarization implicit in the derivation of atomic charges. As shown in Author response image 1, the orientation of the dipole moment calculated with MM (right, blue arrow) is also in good agreement with that predicted with QM (left)



Author response image 1.

> *(1) p. 3 "the last two helices in each subunit" -> "the last two transmembrane helices in each subunit".*

Thanks. Corrected.

> *(2) p. 5 "and therefore do not cause large density variations e.g. 100-fold or greater.". I would be more specific here and indicate what are the actual variations in density or free energy encountered and how they are compared e.g. with thermal fluctuations (~kT).*

Thanks. The exact variations in K+ density had been included in the original manuscript, in Fig. 2C, but we failed to refer to this figure at this point in the description of the results. The ion density is plotted in a log scale to facilitate conversion to free-energy units. Corrected.

> *(3) p. 6 Figure 1 caption "and along the perpendicular to the membrane" -> "perpendicular to the membrane normal"?. "The channel is an assembly of four distinct subunits (in colors);" -> "The channel is an assembly of four identical subunits (distinct by colors);". I would use the same protein coloring method in panels B and C as was used in panel A.*

Thanks. Corrected as needed.

> *(4) p. 6 Figure 2 In panel B I would appreciate a representative complete ion permeation event trace. In panel C caption I would indicate corresponding sites "S0-S4, Scav" for each residue mentioned. I also would not use gray color for site names in the figure.*

We appreciate the suggestion, but believe the figure is clear as is. Panel B is meant to focused on the mechanism of knock-on. Panel A includes numerous complete permeation events.

> *(5) p. 7 Figure 3 caption. Please indicate which atoms of residues T373 and P406 were used to define SF and gate positions. Chemical structures of both TEA and RY785 would*

> *be useful. In panels C and F channel interacting residues (if any) would be helpful to show.*

The revised caption clarifies that the positions of T373 and P406 are represented by their carbonalpha atoms. A close-up view of the structures of TEA and RY785 is included in the Supplementary Information section.

> *(6) p. 8. Figure 4 caption. Please indicate if N atoms ere used for density maps in panels B and C, and which value of the density was used to show meshes. In panel A please indicate what are the units of the density shown by color maps.*

The caption has been revised to clarify these questions.

> *(7) p. 9 "inside the protein" -> "inside the channel pore".*

Thanks. Corrected.

> *(8) p. 10 "which lines the cavity" -> "which lines the water-filled cavity"*

We appreciate the suggestion but believe the wording is clear as is.

> *(9) p.10 Fig. 5. It would be helpful to distinguish residues from different chains e.g. by different colors rather than using different colors for different residues. The S atom in RY785 is hard to recognize due to the yellow color used for C atoms. Figure 5B is very confusing. It is not clear what this plot represents. For instance, what does it mean that Pro405 has ~10 contacts in 20% of simulation snapshots? Does it mean 10 C..C/S interactions within 4.5 A? I am not sure what the value of this is. I think a bar or radar chart plot showing % of contacts with one, two, or more residues of each type would be more helpful.*

Thanks. The revised caption ought to clarify how to interpret the plot.

> *(10) p. 12 "Due to its 2-fold molecular symmetry". TEA has a tetrahedral point group or Td symmetry. It has several two-fold rotational axes though.*

Thanks. Corrected.

> *(11) p. 12 "it prevents K+ ions in the cytoplasmic space from destabilizing the K+ ions that reside in the selectivity filter" I am not sure if this statement is entirely accurate as there might be destabilization of a multi-ion SF configuration not ions per see.*

We believe this statement is clear as is.

> *(12) p. 13 Fig. 7 caption "includes non-conductive or transiently inactivated states" - I am not sure what "transiently inactivated state" is as inactivation is a specific term used in ion channel research and it does not seem to be explicitly considered in this study.*

A reference has been included in the caption for readers interested in the process of inactivation.

> *(13) p. 14 "the net charge of these constructs is thus zero". This would depend on the number of basic and acidic residues in the protein.*

Yes, it does – and as a result the construct we model has a net zero charge.

> *(14) p. 14 I wonder if the protein was constrained or heavily restrained during MARTINI membrane building and equilibration procedure. Otherwise, C-alpha mapping would be problematic and clashes with lipid membrane atoms might take place as well.*

It was indeed. When a protein is simulated using the MARTINI coarse-grained forcefield, its fold must be preserved through a network of strong 'virtual' bonds between adjacent carbon-alpha atoms. This is standard practice so we do not believe it requires further explanation.

> *(15) p. 15 PME - please spell out and provide reference.*

Corrected.

> *(16) p. 15 "with a smooth switching function" - is it a special or standard switching function? Also, was it used for energy or forces?*

The switching function brings both forces and energies to a value of zero at the cut-off value, smoothly. We refer the reviewer to the NAMD manual for further details.

> *(17) p. 15 'k = 1 kBT.' Please confirm that there is a factor of "1" there, which can be actually skipped if this is the case.*

The value of k = 1 KBT is correct.

> *(18) p. 15. Please cite PMID: 22001851 for the transmembrane electric field application technique.*

Corrected.

> *(19) p. 15 "and CHARMM36m" -> "and CHARMM36m force field".*

Corrected.

> *(20) p. 16 "the four proteins subunits" -> "the four protein subunits".*

Corrected.

> *(21) p. 16. Please provide the reference for CGenFF. It's reference 49.*

Corrected.

> *Supporting Information (SI): CGenFF is misspelled in multiple figure captions in the SI. All potential energy scans indicate "angle", but some are bond angles while others are dihedral angles. Using subscripts for atom numbers is confusing and does not match the numbering scheme used in Fig. S1. So, please use the same style of numbering throughout, e.g. C46-C42-N43 (without subscripts). Please label the X and Y axes in Figsures S2-S19 and S21. In Figure S22 please perform a linear regression analysis and/or compute Pearson correlation coefficients and indicate trend lines. Table S1. It would be good to compute RMS or mean unsigned errors to get an idea about accuracy. Also, please indicate if reference QM values were scaled by 1.16 for energies or offset for distances.*

The Supplementary Information has been corrected. We thank the reviewer for their detailed feedback.

> ***Reviewer #3 (Recommendations for the authors):***
>
> *(1) The study needs to consider the possibility of multiple binding sites for RY785, particularly given its impact on voltage sensors and gating currents. Specifically, the potential for allosteric binding sites in the voltage-sensing domain (VSD) should be assessed, as some allosteric modulators with thiazole moieties are known to bind VSD domains in multiple voltage-gated sodium channels (Ahuja et al., 2015; Li et al., 2022; McCormack et al., 2013; Mulcahy et al., 2019). Molecular docking and/or MD simulations could quickly test this hypothesis. If this hypothesis is not true, a comprehensive search*

> *can exclude such a possibility, which can also confirm the long-range allosteric coupling between RY785 binding in the central pore and voltage-sensing domain dynamics.*

Please see our response above.

> *(2) The authors describe RY785 as a selective inhibitor of Kv2 channels and characterize its binding residues through MD simulations. To support this claim, Figure 5 needs to include a multiple sequence alignment with other Kv channels. This would help demonstrate whether the identified RY785-binding residues are indeed unique to Kv2 channels.*

Please see our response above.

> *(3) The study applies a biasing potential to ϕ, ψ, and $\chi 1$ dihedral angles. Please clarify:*
>
> *(a) Is this potential solely to prevent selectivity filter collapse/degradation, as mentioned in a previous D. E. Shaw Research publication (Jensen et al., 2012)?*

Yes, that is correct.

> *(b) If it applies to all amino acids, can this potential prevent other changes, such as in the voltagesensing domain?*

Yes, that is correct.

> *(c) What specific "large-scale structural changes" does this potential preclude?*

For example, it would preclude the spontaneous degradation of the secondary or tertiary structure of the protein. We have revised the Methods section to make these points clearer.

> *(d) Given that such biasing potentials on backbone dihedral angles can decrease conformational flexibility, and considering that Kv channel permeability/conductivity could be highly sensitive to filter flexibility, what insights can you provide about the impact of the force constant k on channel conductivity?*

In previous studies based on an identical methodology (Stix et al, 2023; Tan et al, 2022), we have observed good agreement between calculated and experimental conductance values – at least as good as can be hoped for, when all approximations are considered. Based on the data presented in those studies, we have no reason to believe our methodology inhibits the permeability of the channel, which is logical as the local structural fluctuations required for K^+ flow across the selectivity filter are not impaired, by definition. To the contrary, the fact that these weak biasing potentials make the conductive form of the filter the most favorable state in simulation enable a clear-cut analysis of conductance under plausible simulation conditions, both in terms applied voltage and K^+ concentration. We refer the reviewer to the abovementioned studies for further details and a discussion of this subject.

> *(4) The observation that the Kv2.1 central pore remains partially permeable to K^+ ions when RY785 is bound is intriguing. Given the compact nature of the central cavity when RY785 is bound, it would be valuable to investigate whether polar groups of RY785 (e.g., nitrogens from the amide, benzimidazole, and thiazole moieties) always interact with K^+ ions. Characterizing these interactions could inform the design of similar compounds with differential modulation effects.*

We examined this possibility and detected no convincing interaction patterns between RY785 and K^+ ions – logically, inhibitor and ions are in close proximity while residing concurrently within the pore, but we detected no evidence of specific interactions.

> *Minor points:*

> *It is strongly recommended that the refined force field parameters for RY785 be shared as a separate supplementary file in CHARMM force field format. This addition would be valuable for the scientific community, allowing other researchers to use or compare these parameters in future studies.*

We agree entirely. Upon publication of the VOR for this article the forcefield parameters for RY785 will be made freely available for download at *https://github.com/Faraldo-Gomez-Lab-atNIH/Download*.

> *The study uses a KCl concentration of 300 mM, which exceeds typical intracellular K+ levels. While this may be intentional to enhance K+ permeation probability, a brief justification for this choice should be included in the Methods section.*

Yes, what motivated this choice in this and in our previous studies of K+ channels was the expectation of a greater number of permeation events, for a given simulation length, and therefore greater confidence (i.e. statistical significance) in the observed ion conductance, or in the degree to which it might be inhibited by a blocker. It worth noting that 300 mM KCl, while atypical in the intracellular environment, is often used in electrophysiological studies. The Methods section has been amended to clarify this point.

https://doi.org/10.7554/eLife.101855.2.sa0