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The Molecular Mechanism by which PIP₂ Opens the Intracellular G-Loop Gate of a Kir3.1 Channel

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ABSTRACT Inwardly rectifying potassium (Kir) channels are characterized by a long pore comprised of continuous transmembrane and cytosolic portions. A high-resolution structure of a Kir3.1 chimera revealed the presence of the cytosolic (G-loop) gate captured in the closed or open conformations. Here, we conducted molecular-dynamics simulations of these two channel states in the presence and absence of phosphatidylinositol bisphosphate (PIP₂), a phospholipid that is known to gate Kir channels. Simulations of the closed state with PIP₂ revealed an intermediate state between the closed and open conformations involving direct transient interactions with PIP₂, as well as a network of transitional inter- and intrasubunit interactions. Key elements in the G-loop gating transition involved a PIP₂-driven movement of the N-terminus and C-linker that removed constraining intermolecular interactions and led to CD-loop stabilization of the G-loop gate in the open state. To our knowledge, this is the first dynamic molecular view of PIP₂-induced channel gating that is consistent with existing experimental data.

INTRODUCTION

Inwardly rectifying K⁺ channels (Kir channels) allow greater K⁺ flow into the cell than out of it. Kir channels are composed of seven subfamilies (Kir1–7) and play important physiological roles in a variety of cells. All Kir channels exhibit sensitivity to the signaling phospholipid phosphatidylinositol 4,5 bisphosphate (PIP₂) (1). Numerous experiments have indicated that PIP₂ controls the channel gating mechanism mainly through specific electrostatic interactions (2). Channelopathies (e.g., Barter syndrome and Andersen syndrome) are associated with mutations on Kir channels that affect channel–PIP₂ interactions (3). In the past few years, the publication of several crystal structures of Kir channels has paved the way for understanding the PIP₂-controlled channel gating mechanism at an atomic level of resolution (4–9).

Kir channels, like other K⁺ channels, have a similar tetrameric architecture, but, unlike other channels, they have a long ion permeation pathway that consists of both a transmembrane and a cytosolic portion. Below the transmembrane pore, the permeation pathway is extended by large C-termini forming a cytoplasmic pore. Three relatively narrow regions exist along the permeation pathway: the selectivity filter and the helix bundle crossing (HBC; both in the transmembrane pore), and the G loop (in the cytoplasmic pore). Experimental evidence indicates that these three regions may function as gates to control ion permeation in Kir channels.

High-resolution crystal structures have been reported for a Kir3.1 chimera composed of the prokaryotic Kir3.1 channel (mainly transmembrane portion) and the mouse Kir3.1 (mainly cytosolic portion) (5). These crystal structures revealed two conformations of the cytoplasmic domains, one with a constricted cytosolic (or G-loop) gate (presumed closed conformation) and another with a dilated G-loop gate (presumed open conformation).

The Kir3.1 chimera was successfully reconstituted in planar lipid bilayers (10). The results showed that the Kir3.1 chimera displayed a requirement of PIP₂ for activation of the channel and Mg²⁺–dependent inward rectification. On the other hand, unlike other members of Kir3 subfamily, which require both PIP₂ and Gβγ subunits (or PIP₂ and Na⁺ ions) for activity, PIP₂ alone was able to activate the Kir3.1 chimera. Thus, this Kir3.1 chimera offered a good model for studying structure-function relationships for PIP₂ gating of Kir channels.

Eleven crystal structures, mainly of the bacterial KirBac3.1 channels, revealed two distinct conformations of the cytoplasmic interface between two adjacent subunits (involving the N-terminus of one subunit and the βM sheet of the other), termed latched and unlatched (7). Transition from the latched (closed) to the unlatched (open) conformation occurred through a staged path for each of the four interfaces, and only when all four interfaces adopted unlatched conformations could the channel reach the open state (7).

Previous studies on Kir channels identified a series of key residues involved in PIP₂ sensitivity. Mutation of these residues seemed to weaken the interaction between PIP₂ and the channel, and decrease the channel current (3,11,12). Computational modeling studies predicted the PIP₂ binding mode within the Kir channels and are consistent with experimental data (13). Also consistent with previous electrophysiological and computational results, recent crystal structures of Kir2.2 and Kir3.2 in complex with PIP₂ confirmed sites of interaction, capturing the channel gates in an open or closed conformation (8,9). However, despite...
these advances, it remains unknown how PIP$_2$ induces the conformational change of the channel from the closed to the open state. Here, we used crystal structures of the Kir3.1 chimera to conduct molecular-dynamics (MD) simulations on the constricted conformation in the absence (constricted-apo) or presence (constricted-holo) of PIP$_2$, and on the dilated conformation in the absence (dilated-apo) or presence (dilated-holo) of PIP$_2$. The MD trajectories of each system provided us with detailed information about the conformational changes and the interaction network within these systems. The constricted-holo simulations revealed an intermediate state between the closed and open conformations that allowed us to gain mechanistic insight into the dynamic process of channel gating by PIP$_2$.

MATERIALS AND METHODS
Modeler v9.5 (14) was used to build the missing residues in the crystal structures of the Kir3.1 chimera (PDB entry: 2QKS). We energy-minimized both dilated and constricted structures using the CHARMM program with the implicit membrane/solvent generalized Born (GB) model (15). AUTODOCK (16) was implemented for docking studies. Because PIP$_2$ is too large for a flexible docking procedure, we docked diC1 (an analog to PIP$_2$) into the Kir3.1 chimera structure, and then constructed the PIP$_2$-Kir3.1 chimera complex based on the docked diC1-Kir3.1 chimera complex structure (see Fig. S4 in the Supporting Material for the final docked diC1-Kir3.1 chimera). The complex of PIP$_2$-Kir3.1 chimera was immersed in an explicit lipid bilayer. After each system was solvated and neutralized, we conducted 100-ns MD simulations using GROMACS v4.0.5 (17). Details regarding the methods used for the docking and MD simulations are provided in the Supporting Material.

RESULTS
Dilation of the G-loop gate is triggered by PIP$_2$
Consistent with the KirBac3.1 results (7), the constricted and dilated conformations of the crystal structure of the Kir3.1 chimera showed two key features characteristic of an open channel conformation (7): 1), weakening of a salt-bridge interaction between R313 and E304 of two adjacent G-loop gates; and 2), a large movement of the C-terminal LM loop upward, engaging the N-terminal $\beta$A loop of its adjacent subunit to form an unlatched interface (Fig. S1, A–D; for the nomenclature used for secondary structural elements and reference to specific residues, see Fig. S7). Because PIP$_2$ is sufficient to gate the Kir3.1 chimera (10), we performed 100-ns-long MD simulations after PIP$_2$ was docked onto either the constricted or dilated channel structures (see Materials and Methods), in an attempt to gain insight into the dynamic transition of the cytosolic gate from a closed to an open conformation. We first focused on the G-loop gate variation movements throughout the MD simulations for each of the four systems we studied (constricted-apo, constricted-holo, dilated-apo, and dilated-holo; Fig. 1 and Fig. S2; also see Movie S1, Global Motions). The minimum distances of G loop were

![Constricted Apo](A) (B) (C)
![Constricted Holo](D) (E) (F)
![Dilated Holo](G) (H) (I)

**FIGURE 1** G-loop gate movements in three simulation systems. (A–C) The G-loop gate in the constricted-apo system. (A) The cytoplasmic domain at the end of the simulation viewed from the extracellular side. The transmembrane domain is removed to reveal the G-loop gate from an extracellular view. (B) Ca$^+$ trace of the G-loop gates from each of the four subunits (A–D) as shown in panel A. (C) Side view of the G-loop gate from the diagonal subunits A and B. Similarly, D–F represent equivalent panels as in A–C, but for the constricted-holo system, and G–I represent equivalent panels as in A–C, but for the dilated-holo system.
calculated between the atom center of mass of residues T305–T309 across diagonal subunits of the channel, which equilibrated near 5.5 Å (constricted) or 8.2 Å (dilated). Because the dilated-apo system was stable at the larger minimal distance for 90% of the simulation length and only began to transition to the constricted state at the end of the simulation (Fig. S2 F), we focused on the other three systems for which the conformations were equilibrated for the majority of the simulation time. Interestingly, a PIP$_2$-induced decrease in the root mean-square deviation (RMSD) throughout the simulation trajectories for the four systems suggested that the presence of PIP$_2$ served to stabilize the channel protein (Fig. S2 H). The constricted-apo and constricted-holo systems started with the G-loop gate completely closed. The sulfur atoms of M308 constricted the G-loop gate to a diameter of 5.3 Å. At the end of the simulations, the G-loop gate in the constricted-apo structure turned to asymmetric and appeared in a rectangular-like shape (with subunits A and B ~6 Å apart, and subunits A and D ~11 Å apart; Fig. 1, A and B). In the constricted-holo system, the G-loop gate appeared to be dilated, maintaining almost a fourfold symmetry (Fig. 1, D and E). In the dilated-holo system, the G loops from the last snapshot of the simulation appeared rotated and very similar to the initial dilated crystal structure (Fig. 1, G and H). Side views of two diagonal subunits in each of the three systems (constricted-apo to dilated-holo) showed a progressive widening of the G-loop backbone (Fig. 1, C, F, and I). Thus, the simulations revealed stable conformations distinct from either the constricted or dilated crystal conformations.

The transition of the N-termini implicated in the simulations

We proceeded to conduct a combined principal components analysis (PCA) using a trajectory that was concatenated by the equilibrated trajectories of the constricted-apo and constricted-holo systems (17). The combined PCA is used to depict the collective motions of proteins from one conformational state to the other. In this case, the combined PCA of the first eigenvectors indicated the conformational change of the N-terminus induced by PIP$_2$ in the constricted form. The transition from the constricted-apo to the constricted-holo (Fig. 2, arrows indicate direction) described concerted motions of the N-terminus (residues V55–G58) in one subunit and the βM sheet in the adjacent subunit causing them to interact with one another (Fig. 2 A and Movie S1, Global Motions).

As discussed above, the constricted form of the Kir3.1 chimera has four partially disordered N-termini resembling the latched conformation, as described by Clarke and colleagues (7) for KirBac3.1 (Fig. S1 C), whereas the dilated form of the Kir3.1 chimera has four N-termini forming stable βA contacts with the βM of the adjacent subunit that is aligned in parallel, resembling the unlatched conformation of KirBac3.1 (Fig. S1 D). We proceeded to further examine the interaction observed in the combined PCA between the βM sheet C-terminal interactions with the N-terminal βA. We carried out a defined secondary structures of proteins (DSSP) analysis to monitor the secondary structural variation of residues V55–G58 in each system.

FIGURE 2 N-terminal motions throughout the simulation time that result in the formation of unlatched interfaces. (A) Combined PCA based on the constricted-apo and -holo trajectories. The related regions of the N-terminus, βM, and βL are highlighted in the figure. The transition (arrows indicate direction) from a constricted-apo to a constricted-holo system, leading to the formation of the βA/βM sheet, is shown. (B) DSSP analysis conducted on the three systems throughout the 100-ns simulation time. Only N-terminal residues N54–L60 are plotted in the figure. Each vertical line denotes parallel sheet formation (unlatched interface) in the structure.
during the 100-ns simulation time (Fig. 2 B). The constricted-apo system had two of the four N-termini forming \( \beta A \) sheets, which were loops in the initial structure, parallel to \( \beta M \). The constricted-holo had three N-termini forming \( \beta A \) sheets parallel to \( \beta M \), and the dilated form in the presence of PIP2 kept all four \( \beta A \) sheets parallel to \( \beta M \), as was the case with the initial dilated structure. In other words, the constricted channel in the absence of PIP2 showed two unlatched interfaces, stabilized at a semi-unlatched state. Binding of PIP2 in the constricted channel conformation led to stepwise unlatching of one additional channel subunit interface, and in the dilated conformation, all four interfaces became unlatched.

An examination of interactions at the atomic level in the \( \beta A/\beta M \) sheet showed backbone-backbone hydrogen bonds forming between different strands (involving Q56 and G58 located in \( \beta A \), and F338, V340, and Y342 in \( \beta M \)) that stabilized the \( \beta A/\beta M \) interface. Statistics on such hydrogen bonds for each subunit interface during the simulation time for each system also led to the same conclusions as the DSSP analysis (summarized in Fig. S3 and Table S1).

Thus, the transition from the latched to the unlatched interface between adjacent subunits driven by PIP2 led to changes in the G-loop gate from a constricted fourfold symmetric shape (closed) toward a rectangular asymmetric shape (semi-open) approaching a twofold symmetry, to a square fourfold symmetric shape (open). Given these global effects seen in the presence of PIP2, where the constricted-holo system yielded conformations between the constricted (latched) and dilated (unlatched) ones, we proceeded to examine the detailed PIP2-induced interactions that resulted in the opening of the G-loop gate.

**PIP2 binding site**

Four PIP2 molecules were docked onto each of the subunits at the interface between the transmembrane domain and the cytoplasmic domain, mainly involving the C-linker and the slide helix. Similarly to the PIP2 interacting residues seen in Kir2.2 and Kir3.2 crystal structures (8,9), docking and MD simulations predicted a binding region that included a cluster of similar positively charged residues (Fig. S4 compares our model with the Kir3.2- and Kir2.2 PIP2-ligated crystal structures). Mutation of these residues has been shown to affect PIP2 sensitivity experimentally (3,11). The binding pockets in the constricted and dilated conformations were essentially identical in the initial crystal structures.

After the 100-ns MD simulations, PIP2 was still stabilized at its binding sites in both the constricted (Fig. 3, A and B) and dilated (Fig. 3, C and D) forms. However, the interacting positively charged residues were not identical. On one hand, as shown in Table 1, the N-terminal residues K49, R52, and the C-linker residue R190 interacted with PIP2 only in the constricted form, with R52 forming a stable salt bridge during the simulation. On the other hand, R66 on the N-terminal side of the slide helix, K183 at the cytosolic end of the inner helix, and R219 located on the CD loop interacted with PIP2 predominantly in the dilated form. In contrast, K79 located in the C-terminal side of the slide helix, K188, and K189 located in the C-linker interacted strongly with PIP2 throughout the simulations in both the constricted and dilated forms, although PIP2 overall decreased its interactions with K79, and increased its interactions with K188 and K189 during the transition from the constricted to the dilated conformation. Again, the transient nature of interactions seen between the constricted-holo and dilated systems further reinforced the notion that the constricted-holo conformation represents an intermediate state between the closed and open G-loop states.

**Network of PIP2-induced residue interactions**

The structural transition from the closed to the open state must be accompanied by changes in the network of
interactions, with the formation of new interactions and disruption of old ones. We therefore monitored all hydrogen bonds, salt bridges, and hydrophobic interactions within each system during the 20- to 100-ns simulation time (i.e., the equilibrated portion of the MD trajectories). We first focused on following changes in residue interactions throughout the three systems, seen as a result of the unique interactions occurring in the presence of PIP2 (see Movie S2, Interactions). Percentages of interactions (survival percentage values for a given interaction) were calculated and used to make comparisons among the systems (see Table S1). For clarity, we show in the following figures specific interactions for each system in the last snapshot of the MD simulations.

### Constricted-apo system: the role of the N-terminus in stabilizing the CD loop

For the constricted-apo system, the latched interface between subunits B and C (Fig. 4, blue and gray) is shown to illustrate the critical region of interactions. E304 (of subunit C) is a critical residue of the G loop that formed strong salt-bridge interactions with R313 (of subunit B; Fig. 4 A). As with KirBac3.1 (7), this intersubunit salt-bridge interaction stabilized the closed conformation of the G-loop gate. Weak side-chain–backbone intrasubunit interactions were also seen between R190 of the C-linker and R313. Furthermore, R190 showed predominantly side-chain–backbone and some side-chain–side-chain intrasubunit interactions with residue N50 of the N-terminus. Thus, a critical network of interactions stabilized the closed G-loop conformation: the G loop of each subunit was stabilized through a salt-bridge interaction with the base of the G loop of another subunit, which in turn was stabilized through interactions with its own N-terminus and C-linker.

A second feature of the latched conformation in KirBac3.1 recapitulated in the Kir3.1 chimera was the loose side-chain intersubunit contacts of the N-terminus at Q56 of one subunit with the βM residue F338 of its adjacent subunit (Fig. 4 C). These loose side-chain contacts between the N-terminus and βM in the absence of PIP2 are critical for controlling the CD loop through R219 (see below). Fig. 4, B and C, show stabilization of the CD loop in the inactive conformation by N-terminal residues and R66, located at the N-terminal end of the slide helix. First, the N-terminus was intimately connected with the CD loop through several residue interactions: C53 (of subunit

### TABLE 1 Residues that form salt bridges with PIP2

<table>
<thead>
<tr>
<th></th>
<th>Constricted</th>
<th>Dilated</th>
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<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>K49</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>R52</td>
<td>3</td>
<td>41</td>
</tr>
<tr>
<td>R66 (inter)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>K79</td>
<td>84</td>
<td>48</td>
</tr>
<tr>
<td>R81</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>K183</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>K188</td>
<td>26</td>
<td>1</td>
</tr>
<tr>
<td>K189</td>
<td>75</td>
<td>86</td>
</tr>
<tr>
<td>R190</td>
<td>29</td>
<td>3</td>
</tr>
</tbody>
</table>

Survival percentages of salt-bridge interactions were calculated from 20- to 100-ns-long MD simulations.
B) showed hydrophobic interactions with V224 (of subunit C; Fig. 4 B), whereas C53, V55, and H57 (of subunit B) formed hydrogen bonds with R219 (of subunit C; Fig. 4 C). Second, intersubunit side-chain–backbone interactions between R66 of subunit B with R219 and N220 of subunit C further stabilized the CD loop in the inactive conformation.

The CD loop interacted with the G loop through backbone–backbone intrasubunit hydrogen bonds between S225 and I302 stabilizing the G-loop gate in the closed conformation in the absence of PIP2 (Fig. 4 B). Interactions of three critical CD-loop residues (V224-C53, R219-H57, and N220-R66) are highlighted in a morph movie in the Supporting Material (Movie S2, Interactions).

**Constricted-holo system: a PIP2-induced reorientation of the CD loop**

As we have already considered (Figs. 1 and 2), the constricted-holo system exhibited a PIP2-driven partial dilation of the G-loop gate with three of the four interfaces in the unlatched conformation. The latched and unlatched interfaces exhibited distinct interaction patterns in transition from those seen in the closed and open conformations, and are discussed separately. At the latched interface (Fig. 5, A–C) forming between subunits A and C (pink and gray), the N-terminus was directly affected by PIP2 due to the salt bridge between R52 and PIP2 (Fig. 5 A). This interaction reoriented the N-terminus such that the interaction network among the CD loop, the N-terminus, and the G loop was altered compared with the network in the constricted-apo system (compare Fig. 5, A and B, with Fig. 4, A and B). The N-terminus directly interacted with the G loop (C53 hydrogen bonding with E304), and its interactions with the CD loop were weakened (decreased survival percentages of C53-V224 and C53/V55/H57-R219; see Table S1). The reorientation of the N-terminus and its interactions with E304 of the G loop began to weaken the intersubunit salt-bridge interactions between R313 and E304. Although it is distinct from the constricted-apo form (Fig. 4 C), we consider this interface to be latched, because the N-terminus has not yet formed a stable βA parallel interaction with βM (Fig. 5 C).

From the remaining three unlatched interfaces, the interface between subunits B and D (Fig. 5, blue and yellow) is shown as representative (Fig. 5, D–F). R52 in these subunits continued to form salt-bridge interactions with PIP2. R190 also formed salt-bridge interactions with PIP2, whereas its interactions with N50 were weakened. The N-terminus of subunit D formed stable backbone–backbone hydrogen bonds with the βM of subunit B, yielding the characteristic unlatched interface. C53 no longer interacted with E304 or V224 (compare Fig. 5, D and E, with Fig. 5, A and B), and the connection between the N-terminus and the CD loop relied on the weak interactions of C53/V55/H57–R219 (Fig. 5 F, Table S1). The R66 interactions with R219 and N220 seen in the constricted-apo system were greatly diminished, further destabilizing the inactive CD-loop conformation and freeing the slide helix to move (see below). Another key set of changes occurred in the interactions between the CD and G loops. V224 instead of S225 of the CD loop formed backbone–backbone intrasubunit hydrogen-bond interactions with I302 of the G loop, whereas S225 of subunit B formed side-chain–side-chain hydrogen-bond interactions with E300 of subunit D (Fig. 5 E, Table S1).

Thus, the latched and unlatched interfaces of the constricted-holo simulations revealed a staged transition from a closed to an open G-loop gate conformation triggered by PIP2. PIP2 interactions with the N-terminal R52, followed by the C-linker R190 interactions with PIP2, set the stage for the N-terminus to move from the latched to the unlatched conformation, destabilizing the inactive CD-loop conformation and allowing its interactions with the G loop to begin stabilizing it in the open (or dilated) conformation (see below). Interactions of R52 and R190 with PIP2, as well as interactions of the CD loop with N-terminal residues, are highlighted in the morph movie (Movie S2, Interactions).

**Dilated-holo system: interactions between the CD and G loops establish the open G-loop conformation**

The dilated-holo system showed all subunits to be in the fully unlatched conformation throughout the simulation time. The interface between subunits B and C (Fig. 6, blue and gray) is used to illustrate the detailed interactions. Fig. 6 A and Table S1 show that the salt bridge between E304 and R313 was further weakened from the constricted-holo system: on one hand, R313 formed side-chain–backbone hydrogen-bond interactions with D48 and G51 of the N-terminus that was now in the unlatched conformation; on the other hand, E304 showed a higher percentage of side-chain–side-chain and side-chain–backbone hydrogen bonds with H222 than in the other systems we studied. We monitored the distance variation between E304 and R313 for all systems throughout the 100-ns simulations, and found that this salt bridge was weakened as PIP2 caused progressively the opening of the G-loop gate (see Fig. S5).

The CD loop in the dilated-holo system closely interacted with the G loop through stable intramolecular backbone–backbone hydrogen bonds between V224 and I302 (Fig. 6 B). At the same time, S225 of subunit C formed side-chain–side-chain hydrogen-bond interactions with E300 of subunit B. This hydrogen-bond pattern was almost identical to that seen in the unlatched interfaces in the constricted-holo system (Fig. 5 E), but was more stable in the dilated-holo system (i.e., a higher percentage of interactions in the MD trajectory of all subunits; see Table S1). A clear difference between these two systems was seen in the interactions of R219 (Fig. 6 C), which showed weak interactions with the N-terminus in the constricted-holo system (Fig. 5 F), and instead interacted with PIP2 in the dilated
conformation. In other words, PIP$_2$ attracted R219, aiding in the disruption of its interaction with the N-terminus and helping to optimize the intrasubunit interactions of the CD loop with the G loop. As the N-terminus lost its intersubunit interactions with the CD and G loops, it moved downward to form stable interactions with $\beta$M and establish the unlatched interface (Fig. 6 C). This reorientation of the N-terminus and CD loop relieved the interactions of R66 with R219 and N220, allowing it to also interact with PIP$_2$ in the dilated conformation. Interactions of R66 and R219 with PIP$_2$, as well as the weakening of the CD loop with the N-terminal residues, are highlighted in the morph movie (Movie S2, Interactions). Interestingly, a comparison of Kir3.2 crystal structures showed an outward movement of the LM loop accompanied by a concomitant slight displacement of the N-terminal $\beta$A (Fig. S6). Thus, the movement of the LM loop in Kir3.2 appears to contrast with that of the LM loop in the Kir3.1 chimera. This marked difference between Kir3.1 and Kir3.2 subunits in the direction of the displacement of the LM loop maybe related to differences in the gating between Kir3.1 and other Kir3 subunits (18).

FIGURE 5 Network of interactions among the G loop, CD loop, $\beta$H, and N-terminus in the constricted-holo system. (A–C) Latched interface between subunits A (pink) and C (gray). (D–F) Representative unlatched interface between subunits B (blue) and D (yellow). Interacting residue pairs are listed below each panel of the figure and the nature of their interactions is denoted (hp, hydrophobic; hb, hydrogen bond; stb, salt bridge; BB, backbone–backbone; SB, side-chain–backbone; SS, side-chain–side-chain).
Other critical interactions in the PIP₂-induced gating transition

The βH and βI strands lead to the G (or HI) loop cytosolic gate on one end, and on the other end βH is connected to the GH loop, and βI is connected to the IJ loop (see Fig. S7 and Fig. S8). A set of interesting PIP₂-dependent interactions involve E294, located at the end of βH, following the GH loop, with the N-terminus (Fig. S8). In the constricted-holo system, E294 formed salt-bridge interactions with R45 of the N-terminus. Interestingly, E318 at the 3–10 helix past the βI strand in the IJ loop formed a salt bridge with R45 in the constricted-apo system. The PIP₂-driven reorientation of the N-terminus is likely driving this molecular switch (R45 interacting with E318 in the absence of PIP₂ but switching to interact with E294 in the presence of PIP₂) to affect the G-loop conformation on the other end of the βH and βI strands (see Table S1).

An interesting shift in the balance of interactions with PIP₂ occurred with the establishment of the R66-PIP₂ salt bridge (see Table 1). The constitutive K79–PIP₂ interaction was decreased (K79 is positioned on the other side of the slide helix from R66). On the other hand, K183 (two residues at the C-terminal end from the narrowest constriction point of the inner helix gate (19)), which formed stable hydrogen bonds with residues V76 and L78 on the C-terminal side of the slide helix (between the side chain of K183 and the backbone of V76/L78) in all three simulation systems, showed a decrease in K183–L78 interactions (see Table S1). Concomitantly, a new interaction was established between K183 and PIP₂. In parallel, another constitutive interaction of the C-linker with PIP₂ (K188) increased. These set of interactions following the effects of PIP₂ on the G-loop gate in the dilated-holo system suggest a coupling of the opening of the G loop toward opening of the inner helix (HBC) gate. Toward the end of the dilated-holo system simulations, we began to see effects in one subunit at the inner helix gate (Fig. S9). Although in this study we focused on the mechanism of the opening of the cytosolic gate, our results with the subsequent effects of PIP₂ on residues flanking the slide helix (R66 and K79), the C-linker (K188), and K183 located next to the HBC gate, suggest that opening of the inner helix gate would follow.

Experimental evidence for the Kir3.1 chimera gating model

The overall gating scheme of opening the G-loop gate appears to be orchestrated by the N-terminus. In the absence of PIP₂, in its latched interface with βM, it keeps the CD loop (with the help from the C-linker) from interacting optimally with the G-loop gate. In the presence of PIP₂, the N-terminus is driven to an unlatched interface with βM that unleashes the CD loop to interact optimally with the G-loop gate. In the presence of PIP₂, the N-terminus stabilized the C-linker (K49- and N50- with R190) and the CD (C53-V224, C53/V55,H57-R219) and LM (Q56-F338) loops. At the same time, the N-terminal
side of the slide helix (R66) interacted with the CD loop (N220 and R219). 2) In the intermediate conformation, PIP2 first engaged the N-terminus (R52), weakening its interaction with the CD loop (C53-V224). The C-linker (R190) followed to also interact with PIP2, helping the N-terminus to alter its interaction with the LM loop (Q56 now interacted with V340 and Y342, and G58 also interacted with Y342). These changes were characteristic of the unlatched interface. As a result, the intersubunit interaction between adjacent G loops (E304-R313) was weakened and the CD-loop interaction with the G loop was strengthened (V224-I302 and S225-E300). In the dilated conformation, PIP2 further stabilized the CD loop by engaging R219 and engaging the N-terminal side of the slide helix through R66, relieving the CD loop to further stabilize the G loop (H222-E304) in the dilated conformation. In addition, the intersubunit G-loop interaction was further weakened (E304-R313), aided by interactions between the C-linker, the N-terminus, and the G loop (G51-R313, K49-R190, and R190-T314).

Given the high conservation among Kir channel structures, we compared existing experimental data against the predictions made by the Kir3.1 chimera gating model. A sequence alignment of several Kir channels facilitates comparisons of key residues that serve similar roles in different channels (Fig. S7).

A mutagenesis study performed on Kir2.1 identified a number of basic residues that altered sensitivity to PIP2 (3). These residues corresponded to Kir3.1 residues K49, R52, R66, K183, K188, K189, R190, and R219. K79, which was identified by our computational study, was not found to affect PIP2 sensitivity in Kir2.1, where it is an Arg residue. Yet, mutation of the residue preceding Kir2.1(R80) to the corresponding Kir3 or Kir6.2 Leu residue (I79L), reduced the stereospecificity of Kir2.1 for PIP(4,5)P2 (12). Thus, Kir3.1 (K79) and surrounding residues may play a key role in positioning the inositol ring to exhibit stereospecific interactions with interacting residues of different Kir channels. Additionally, basic residues that experimentally were identified to affect PIP2 sensitivity in Kir2.1 but were not predicted in the Kir3.1 chimera to interact with PIP2, were R229 and R313. As we have discussed, R313 is critical for stabilizing the closed G-loop conformation, and its mutations are likely to reflect allosteric changes in PIP2 sensitivity. R229 could also be acting allosterically. Thus, there is strong agreement between computational predictions in the Kir3.1 chimera and experimental results in Kir2.1, supporting the notion of a highly conserved gating mechanism in Kir channels.

The critical role of the CD loop in controlling PIP2 sensitivity was recognized more than 12 years ago (11). Key residue differences between Kir2.1 and Kir3.4 were identified (equivalent residues in Kir3.1: R219 and M223). Moreover, Kir3.1 residue N217 (or the corresponding Asn residue in Kir2.1) was shown to coordinate Na+ ions when mutated to Asp residues, just as it does in Kir3.2 and Kir3.4, where it occurs naturally (11,20). Subsequent studies showed that Na+ ions are coordinated by the side chains of the Asp residues and a His residue (Kir3.1-H222) on the other side of the CD loop (21). More recently, the critical roles of this CD-loop His residue and a conserved Val residue in the gating of the Kir2 channel G loop (corresponding to the Kir3.1-V224) and its stabilization at the open conformation were established (H. An and D. E. Logothetis, unpublished). Thus, experimental evidence for the critical roles of the Kir3.1 equivalent residues N217, R219, H222, M223, and V224 has been obtained in a number of Kir channels, strongly supporting a key role of the CD loop in PIP2-dependent gating.

The N-terminal βA movement from the latched to the unlatched conformation is a critical element in the gating of the Kir3.1 chimera, as it is for KirBac3.1. H57 within the βA interacts with R219 in the constricted conformation, but as PIP2 drives the channel to unlatch, the H57–R219 interaction weakens and R219 interacts with PIP2 to stabilize the open state of the G loop. The Kir3.1 mutant H57F abolished the channel activity of a pore-helix mutant that showed homomeric channel activity (22,23). The equivalent Kir3.4 residue also abolished homomeric channel activity, but could be rescued by CD-loop mutations that stabilized interactions with PIP2 (22). The Kir3.1 (C53) residue was shown to underlie activation of the channel by DTT (24). This is the same residue that in the absence of PIP2 was found to interact with the CD-loop V224 residue to stabilize the G-loop gate in the closed state. Mutations of the LM-loop Kir3.2 residue Y349 (corresponding to Kir3.1-F338) either reduced both agonist- and alcohol-induced channel activation or abolished the currents completely (25). Moreover, Y349 with the N-terminal residue Y58 (corresponding to Kir3.1-F46) is involved in coordinating alcohols that stimulate Kir3 channel activity. Kir3.1-F338 in our simulations was involved in the latching conformation of the LM loop with the βA N-terminal helix (Kir3.1-Q56). These experimental results further support the importance of the N-terminal interactions with the C-linker, CD loop, and LM loop in controlling the stability of the cytosolic gate in the closed or open conformation.

**DISCUSSION**

Our results establish the constricted channel conformation in the presence of PIP2 as an intermediate state between the constricted form in the absence of PIP2 and the dilated form in the presence of PIP2. Based on the interactions seen in this intermediate state, we were able to discern the staged path of gating: PIP2 opened the constricted G-loop gate as it drove changes in the interactions of the N-terminus with key elements of the cytosolic gating apparatus, namely, the CD loop and G loop. This global gating transition showed a remarkable resemblance to the gating transitions...
Kir3.1 chimera can be gated by PIP2 alone (10); therefore, G-loop gate but the HBC gate remained closed. The channel (3). The results suggest that the positive charge the expectation is that PIP2 also ought to open the HBC transactional interactions are established. The K183 interaction occurs on a timescale longer than our simulations, as addi- tion in the L60-R66 region that needed to be modeled. Yet, R66 is a critical residue and none of the existing structures reveal its role. Mutations of the equivalent residue in Kir2.1 (R67) to Gln or Cys have been shown to dramatically reduce channel sensitivity to PIP2 (3). Both our models and the crystal structures reveal that the N-terminal side of the slide helix becomes more ordered in the presence of PIP2. However, both the Kir3.1 chimera crystal structures and our simulations with PIP2 differ from the crystal structures in that the slide helix does not bend as it does with the Kir3.2 and Kir2.2 structures in the presence of PIP2. These observations suggest that PIP2 not only causes the N-terminal side of the slide helix to become ordered, it also induces a bend that positions the R66 residue away from PIP2. It is possible that longer simulations would reveal this bending of the slide helix and interrupt the R66 interactions with PIP2, propagating the PIP2 interactions to the C-terminus of the slide helix and K183 to gate the inner helix gate to an open conformation. This hypothesis needs to be further addressed in future work.

In this study we focused on the effects of PIP2 in the gating of the intracellular G-loop gate. Clearly, for ions to permeate through the channel, not only the G-loop gate but also the HBC or inner helix gate would need to open. In all of our simulations, PIP2 was able to open the G-loop gate but the HBC gate remained closed. The Kir3.1 chimera can be gated by PIP2 alone (10); therefore, the expectation is that PIP2 also ought to open the HBC gate. We did not observe this result in our 100-ns-long simulations. It is possible that the opening of the HBC gate occurs on a timescale longer than our simulations, as additional interactions are established. The K183 interaction with PIP2 is suggestive in this way of thinking. Mutagenesis data on Kir2.1 channel indicate that K182Q (equivalent to K183 in Kir3.1) weakened the interaction between the channel and PIP2, whereas K182R caused a nonfunctional channel (3). The results suggest that the positive charge on K182 stabilized the PIP2-channel interactions, whereas the K182R mutant could disrupt the side-chain–backbone hydrogen bonds with V76 and L78 due to the larger side chain of Arg. Similarly, the K182C mutant was nonfunctional. In the Kir3.1 chimera, K183 established its interactions mainly in the dilated-holo system; therefore, it seems that it is a later event in the gating mechanism, in similarity to the behavior of the R66 and R219 residues. It would be interesting to examine whether additional interactions that lead to the opening of the HBC gate would occur in longer simulations. Indeed, a recent structure (9) showed that the Kir3.2-R201A (corresponding to R190 on Kir3.1) mutant that opened the G-loop gate enabled PIP2 to open the inner helix gate (at F192 corresponding to Kir3.1-F181). Together with the new Kir3.2 structure, our data suggest that PIP2 first opens the cytosolic gate, an event that is required for opening of the inner helix gate.

The results of our simulations indicate that the C-linker residue R190 interacted with PIP2 only in the constricted form. Because the C-linker of the constricted form was disordered, we modeled it using the dilated crystal structure as the template. Thus, our confidence in the behavior of the C-linker in the constricted conformation is limited. Yet, we have confidence that in the dilated form, R190 formed stable hydrogen bonds with R313 and T314 located on βl. These interactions likely contribute to the weakening of the conserved salt bridge of E304-R313 (see Fig. 6 A and Table S1). These hydrogen bonds were rarely formed in our modeled C-linker region of the constricted-apo system. Instead, R190 in the constricted-apo conformation was inclined to interact with N-terminal residues K49 and N50 during the simulation. Thus, this predicted transient PIP2 interaction with R190 would serve a dual purpose: first, to weaken its interactions with the N-terminus, and second, to engage R313 and liberate the G loop by contributing to the weakening of its interaction with E304.

The current model makes explicit predictions regarding interactions that stabilize the closed and open conformations. Thus, functional tests of specific mutations that would destabilize one or the other state are needed. Predictions of the Kir3.1 chimera model that have not been previously tested include mutations destabilizing the closed state (E304-R313, R219 with C53/V55/H57, and R219/ N220 with R66), and mutations destabilizing the open state (H222-E304, V224-I302, and S225-E300). Recent structures of wt-Kir3.2 in the presence and absence of Na+ and PIP2, as well as an Ala mutant corresponding to Kir3.1-R190 that causes the G loop to open, offer new possibilities for similar modeling studies that could be followed more readily by means of experimental tests and would allow differences in gating between Kir3.1 and 3.2 channels to be probed. Homology models of the full Kir3.1 structure would also be more amenable to experimental tests. These advances are also pointing us in a direction that may allow
us to discern the relative contribution of distinct gates during channel gating of Kir channels.

**SUPPLEMENTARY MATERIAL**

Supplementary methods, nine figures, a table, two movies, and references are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495(12)00391-8](http://www.biophysj.org/biophysj/supplemental/S0006-3495(12)00391-8).

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**REFERENCES**


