The cytosolic GH-loop regulates the PIP2-induced gating kinetics of Kir2 channels

Hai-Long An1,2, Shou-Qin Lü4, Jun-Wei Li3, Xuan-Yu Meng1, Yong Zhan2, Meng Cui1, Mian Long4*, Hai-Lin Zhang3*, Diomedes E. Logothetis1*

1Department of Physiology and Biophysics, School of Medicine, Virginia Commonwealth University, VA, 23298
2Institute of Biophysics, School of Sciences, Hebei University of Technology, Tianjin, PR China
3Key Laboratory of Neural and Vascular Biology, Ministry of Education; The Key Laboratory of Pharmacology and Toxicology for New Drug, Hebei Province; Department of Pharmacology, Hebei Medical University, Shijiazhuang, PR China
4Key Laboratory of Microgravity (National Microgravity Laboratory) and Center of Biomechanics and Bioengineering, Institute of Mechanics, Chinese Academy of Sciences, Beijing, PR China

Running title: PIP2-induced gating mechanism of Kir channels

*To whom correspondence should be addressed:
Diomedes E. Logothetis, Department of Physiology and Biophysics, School of Medicine, Virginia Commonwealth University, VA, 23298, Tel: (804) 828-5878, Fax: 804 828-7382, E-mail: delogothetis@vcu.edu; or
Hai-Lin Zhang, Key Laboratory of Neural and Vascular Biology, Ministry of Education; The Key Laboratory of Pharmacology and Toxicology for New Drug, Hebei Province; Department of Pharmacology, Hebei Medical University, Shijiazhuang, PR China, Tel: +86-311-86265562, Fax: +86-311-86625562, E-mail: z.hailin@yahoo.com; or
Mian Long, Key Laboratory of Microgravity (National Microgravity Laboratory) and Center of Biomechanics and Bioengineering, Institute of Mechanics, Chinese Academy of Sciences, Beijing, PR China, Tel: +86-10-82544131, E-mail: mlong@imech.ac.cn.

Keywords: gating kinetics; molecular dynamics; patch clamp; two-electrode voltage clamp

Background: The detailed mechanism of PIP2-induced Kir channel gating remains elusive.

Results: Specific mutations increase the flexibility of the cytosolic GH loop and accelerate the PIP2-induced gating kinetics of Kir2 channels.

Conclusion: The GH loop-N-terminal interactions regulate the PIP2-induced gating kinetics of Kir2 channels.

Significance: Identification of a novel region in Kir channels involved in the control of PIP2-induced gating.

SUMMARY

Inwardly rectifying K+ (Kir) channels set the resting membrane potential and regulate cellular excitability. The activity...
of Kir channels depends critically on the phospholipid PIP$_2$. The molecular mechanism by which PIP$_2$ regulates Kir channel gating is poorly understood. Here, we utilized a combination of computational and electrophysiological approaches to discern structural elements involved in regulating the PIP$_2$-induced gating kinetics of Kir2 channels. We identify a novel role for the cytosolic GH loop. Mutations that affect directly or indirectly GH loop flexibility (e.g. V223L, E272G, D292G) increased both the on- and especially the off-gating kinetics. These effects are consistent with a model in which competing interactions between the CD and GH loops for the N-terminus regulate the gating of the intracellular G-loop gate.

Inwardly rectifying K$^+$ (Kir) channels play a vital role in many physiological processes, such as control of heart rate, maintenance of the resting membrane potential, hormonal secretion, extracellular K$^+$ buffering in the brain and K$^+$ secretion in the kidney. These physiological processes are regulated by specific stimuli that control the gating of Kir channels. Examples of stimuli gating Kir channels include intracellular H$^+$ for Kir1 (1), the $\beta\gamma$ subunits of G proteins for Kir3 (2), and PIP$_2$ (phosphatidylinositol 4, 5-bisphosphate) for all Kir channels (3,4). The inwardly rectifying ATP-inhibited K$^+$ (K$_{ATP}$) (Kir6.2) channel was the first reported to depend on PIP$_2$ (5,6). Subsequently, all known Kir channels were shown to depend directly on phosphoinositides for maintenance of their activity (3,5,7-10). In general, Kir channels, as is the case with most ion channels tested, run down when PIP$_2$ is depleted (dephosphorylated by lipid phosphatases) and reactivate when PIP$_2$ is applied from the intracellular side (11). The gating kinetics of Kir channels are quite distinct between different Kir channels but the underlying reasons are not understood.

Kir channels, like other K$^+$ channels, are homo- or heterotetramers of four subunits. Each subunit shares a common structure consisting of a cytosolic N-terminal domain, an outer transmembrane helix (M1), an ion-selective P loop (selectivity filter - SF), an inner transmembrane helix (M2) lining most of the permeation pathway, and a cytosolic C-terminus controlling ion flow (12,13) (Fig. 1A). The SF and bundle crossing of the M2 helix are thought to serve as gates, constriction points along the permeation pathway limiting ion flow (12). Mechanisms of gating (i.e. transitions between the closed and open states) for the SF and M2 gates have been studied and proposed (14). The N- and C-termini, which are located on the intracellular side of the membrane, form a comparable cytosolic extension to the transmembrane portion of the pore. In addition to the SF and M2 transmembrane gates, a cytosolic constriction (HI- or G-loop) of the permeation pathway has been proposed as a third gate (Fig. 1A). Specific acidic residues localized in the cytosolic pore are thought to give rise to inward rectification (15). Functional studies and mutagenesis data have identified several residues as potential PIP$_2$ interacting sites in both the N- and C-termini of the cytoplasmic domain of Kir2.1 channel. Many of these residues are basic and thus capable of forming salt bridge interactions with the negatively charged head group of PIP$_2$ (3,8,10,16). Recent crystallographic evidence has confirmed this view (17,18). Moreover, crystal structures of a Kir3.1 chimera caught the intracellular G loop gate in dilated (open) and constricted (closed) conformations. The
movement in the G loop observed in different structures of the cytoplasmic domains is also notable (9,19). However, it is not known how the G loop gate integrates into channel gating and how it affects the gating kinetics of Kir channels.

Although recent crystallographic studies have greatly enhanced our understanding of the 3-D structure of Kir channels (13,17,20), it has not been possible to understand yet the dynamic gating mechanism of these channels induced by molecules, such as PIP$_2$. A recent study of multiple KirBac3.1 structures in either the open or closed conformations has highlighted the importance of inter-subunit interactions between the N- and C-termini and the G loops of adjacent subunits in Kir channel gating (21).

Here we set out to study Kir channel gating kinetics focusing on the cytosolic channel regions, using a voltage-gated phosphatase to control PIP$_2$ levels (22) and computational, mutagenesis and electrophysiological approaches to probe the role of specific secondary structural elements and specific residues within them. We observed highly correlated movements of the Kir2.1(V223) (of the CD loop) and E272 (of the GH loop) with A306 at the G loop gate (see sequence alignment of Fig. S1). Mutations, at V223 in Kir2.1 or V224 in Kir2.2, accelerated both the opening and closing gating kinetics of the channel and increased the flexibility of the GH loop. Similar results were obtained with mutations of key residues within the GH loop. Our results are explained by a model in which PIP$_2$ induces the switching of the N-terminus from the CD loop to the GH loop (around the short αG helix) (Fig. 1A). Mutations that decrease the GH loop interactions with the N-terminus (e.g., V223L, E272G, D292G) increase the flexibility of the GH loop and allow the N-terminus to gate the G loop with faster kinetics.

**EXPERIMENTAL PROCEDURES**

**Chemicals:**

Phosphatidylinositol-4,5-biphosphate diC8 PIP$_2$ was purchased from Avanti Lipids and was prepared as described previously (8,10). All other chemicals were purchased from Sigma.

**Homology Modeling.** Modeler V9.5(23) was used to add missing residues to the crystal structures of Kir2.1 (PDB entry: 1U4F) and the full-length crystal structure of Kir2.2 (PDB entry:3JYC). Modeler was also used to create a homology model of the cytosolic domain of Kir2.2. The mutant channels were constructed by substituting the WT side chain with the specified side chains. The models were then subjected to at least 3000 steps of a steepest descent minimization using the CHARMM program with implicit membrane/solvent Generalized Born (GB) model (24).

**Molecular docking.** AUTODOCK (25) was used for the docking studies. We replaced PIP$_2$ with its analog (diC1 PIP$_2$), which has two methyl groups. The atomic charges of the PIP$_2$ head group were taken from the ab initial calculations by Lupyan and colleagues (26). A grid map was generated for the Kir2.2 full-length structure using CHNOP (i.e. carbon, hydrogen, nitrogen, oxygen, and phosphor) elements sampled on a uniform grid containing 120×120×120 points, 0.375 Å apart. The center of the grid box was set to the center of known critical PIP$_2$ sensitive residues, i.e. Q51, R65, K183, R186, K188, K189, R190, R219, K220, R229, and R313. The Lamarckian Genetic Algorithm (LGA) was selected to identify the binding conformations of the ligands.
100 docking simulations were performed and the final docked PIP$_2$ analog configurations were selected based on docked binding energies and cluster analysis. The PIP$_2$-Kir2.2 complex was constructed based on the docked PIP$_2$ analog-Kir2.2 complex structure, and refined by CHARMM using the same protocol as described above.

**Molecular Dynamics Simulation (MDS).** The crystal structure of Kir2.1 cytosolic domain were initialized as follows, solvating the molecule in a rectangular water box of 82×104×103 Å$^3$ and neutralizing the water box by adding Na$^+$ and Cl$^-$ of ~ 100 mM. MD simulations were performed using NAMD (http://www.ks.uiuc.edu/Research/namd/) with CHARMM27 all-atom force field parameters (27). An integration time step of 1 fs, a uniform dielectric constant of 1.0, a scaling factor for 1-4 interactions of 1.0, and periodic boundary conditions were applied in all simulations. A smooth (12-16 Å) cutoff and the Particle Mesh Ewald (PME) (28) were employed to calculate van der Waals forces and full electrostatics, respectively. Prior to the equilibration process, energy minimization (5000 steps with backbone atoms of C-terminal fixed and then another 5000 steps with all atoms free), followed by a heating up process from 0 to 300 K over 35ps were performed. Then two 5 ns equilibration processes, with either all atoms free or partly constrained, were performed with the temperature held at 300 K using Langevin dynamics, while the pressure was held at 1 atm using the Langevin piston method. RMSF (root mean squared fluctuation) analysis was performed based on the two equilibration processes. For the full-length Kir2.2 channel simulation, the channels were immersed in an explicit palmitoyloleoyl-phosphatidylcholine (POPC) bilayer generated from the VMD membrane package. After being solvated with SPC water molecules, neutralized by Na$^+$ as the counter ions and including K$^+$ located in the selectivity filter as obtained from the crystal structures, each system involved ~ 141,000 atoms in the MD simulations. GROMACS v4.0.5 (http://www.gromacs.org) was used to conduct the simulation with the GROMOS96 53a6 force field. The force field parameters for PIP$_2$ were generated from the Prodrd server [8] and same atomic charges of the PIP$_2$ head group in the docking step were used in the MD simulations. The lipid parameters were obtained from Tieleman through his website (http://moose.bio.ucalgary.ca). Long range electrostatics were calculated using the PME8 method with 12 Å cut-off. Van der Waals interactions were modeled using Lennard-Jones 6-12 potentials with 14 Å cut-off. All simulations were conducted at a constant temperature of 300K using the Berendsen thermostat. The system pressure was coupled at isotropic (X+Y, Z) directions referenced to 1 bar using the Berendsen method9. All bonds were constrained with the LINCS algorithm10. The time step was 2fs and the neighboring list was updated every 10 time steps.

Prior to production runs, energy minimization of 3000 steps of steepest descent were carried out on each system followed by a 0.5ns two-step equilibration process with either all atoms free or partly constrained. An electric field of 0.06 V/nm was applied in this step as well as the production run, along the z-axis of the box to maintain the lower potentials in the intracellular side. The treatment of the electric field has been detailed in ref (29,30). A 100ns production run was conducted on each system and coordinates were saved
every 10ps for analysis. Visual molecular dynamics (VMD) was used for conformational visualization.

**H Bond Analysis.** The interactions of hydrogen bonds, salt bridges and hydrophobic contacts analysis were calculated using the Simulaid(31-34) program after energy minimization. The Simulaid outputs for interactions were reorganized with in-house scripts for facility of comparison among the systems.

**Molecular Biology.** All cDNA constructs were subcloned into the pGEMHE plasmid vector and used as described. Point mutants were produced by Pfu mutagenesis with a Quickchange kit (Stratagene). Sequences were confirmed by DNA sequencing. Recombinant Kir2.1, its mutants and Ci-VSP were expressed in *Xenopus laevis* oocytes as previously described(10,35). cRNA was produced with T7 RNA polymerase using a kit (Promega). cRNAs of the various Kir2.1, Kir2.2 and their mutants and of Ci-VSP were injected in the range of 0.5-10 ng/oocyte depending on the functional expression level of the given construct.

**Electrophysiology.** Recordings in *Xenopus laevis* oocytes were performed 1–2 days following cRNA injection. Whole-oocyte currents were measured by conventional two-electrode voltage clamp (TEVC) using a GeneClamp 500 amplifier (Molecular Devices, CA). Electrodes were filled with 3 M KCl dissolved in 1% agarose to prevent the leakage of KCl into the oocytes. The electrodes had resistances less than 1 MΩ. Oocytes were constantly perfused with a high-potassium solution (ND96K) containing in mM: 96 KCl, 1 NaCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES (pH 7.4 with KOH). A low-potassium solution (ND96) was used in some experiments to inhibit most of the Kir2.1 currents at -80 mV. To activate Ci-VSP, the following voltage protocol was used in TEVC oocyte recordings: 1s-sweeps composed of a 170 ms ramp from -80 to +80 mV followed by an 830 ms step to +80 mV. To deactivate Ci-VSP, the oocytes were held at -80 mV. Sweeps were applied till the resulting currents reached steady state. ND96 contained in mM 96 NaCl, 1 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES (pH 7.4 with NaOH). Current amplitudes were measured at +80 mV and -80 mV. Data acquisition and analysis were achieved using pClamp9.2 (Molecular Devices, CA) and Origin 7.5 (Microcal, MA) software.

Macropatch channel activity was recorded from devitellinized oocytes under the inside-out mode of standard patch-clamp methods, using an Axon 200B patch-clamp amplifier and Clampex 10.0 data-acquisition software (Molecular Devices, CA). Electrodes were made from borosilicate glass using a Sutter P-97 microelectrode puller (Sutter Instrument Co., CA) that gave a tip diameter of 5-15 μm and had a resistance of 0.5-1 MΩ when filled with an electrode solution containing in mM: 120 KCl, 2 MgCl₂, and 10 HEPES (pH 7.4 with KOH). Two bath solutions were used: (1) a FVPP solution in mM: 60 KCl, 5 EDTA-K, 5 KF, 0.1 Na₃VO₄, 10 K₃P₂O₇ and 10 HEPES (pH 7.4 with KOH). (2) FVPP solution with X M (0-100 M for dose response and 30 M for gating kinetics measurements) diC8 PIP₂. Data were analyzed using pClamp9.2 (Molecular Devices, CA) and Origin 7.5 (Microcal, MA) software.

**HA Tag Expression Experiments.** We engineered an HA tag to the extracellular loop between M1 and the P region of Kir2.1 (8). Oocytes were blocked for 60 min in ND96 with 1% bovine serum albumin (BSA) at 4°C, labeled with 0.5 μg/ml rat
monoclonal anti-HA antibody (3F10; Boehringer Mannheim) (in 1% BSA for overnight at 4°C), washed at 4°C, and incubated with HRP-coupled secondary antibody (goat anti-rat) (in 1% BSA for 60 min). Cells were washed (1% BSA, 4°C) and transferred to ND96 without BSA. Individual oocytes were placed in 55 μl SuperSignal ELISA (Pierce) and incubated at room temperature for 1 min. Chemiluminescence was quantified in a TD-20E luminometer (Turner Designs, CA). As a control, we used uninjected oocytes.

Data Analysis. Error bars in the figures represent S.E.M. Each experiment shown or described was performed on 5-7 oocytes of the same batch for TEVC recording and 3-5 oocytes of the same batch for macropatch recordings. A minimum of 3-4 batches of oocytes was tested for each experiment shown.

RESULTS
The V223 and E272 show highly correlativity with A306 in the G loop gate. To explore the mechanism by which the G loop gate relates to the rest of the cytosolic domain during PIP$_2$-induced Kir channel gating, we performed 10ns molecular dynamics (MD) simulations of the cytosolic domains of the WT Kir2.1 and analyzed the relative movements between C-terminal residues and the G-loop residue A306. From the free MD simulations we found that the relative movements between E272 (at the beginning of the GH loop), A306 (at the apex of G loop) and V223 (in the CD loop) were highly correlated (Fig. 1B). Figure 1C shows the location of residues V223, E272 and E306 in the cytosolic portion of the crystal structure. Unlike the CD and G loops that are physically proximal and are thought to interact with each other, the GH loop is located quite far from the other two loops. We hypothesized that V223 and E272 could play important roles in the PIP$_2$-induced Kir channel gating.

The Kir2.1(V223L) and Kir2.2(V224L) affect the PIP$_2$-induced gating kinetics through a common mechanism. We first examined the effect of mutations at the Kir2.1 223 position from Val to Leu or Met. Figure 2A shows a right shift of the dose-response curve of V223L responding to the stimulatory effect of PIP$_2$ (EC$_{50}$ for WT: 2.75±0.12μM and for V223L: 8.57±0.76μM). However, unlike other mutants reported to decrease the apparent affinity to PIP$_2$, the V223L mutant displayed a 20% increase in its peak currents (Fig. 2B). We next measured membrane surface expression of the WT and V223L mutant, using HA-tagged channels. Our data indicated a 42% reduction in cell surface expression for V223L (0.58±0.01, when WT Kir2.1 was normalized to 1. These results suggested that the increased whole-cell currents of the V223L mutant were not due to an increase in available channels on the cell surface but likely to increased activity. To further examine this possibility, we measured the gating kinetics of Kir2.1 and its V223L mutant, using either two-electrode voltage clamp (TEVC) and the lipid phosphatase Ci-VSP (Ciona intestinalis voltage-sensitive phosphatase) (22) in intact cells or inside-out patches of the patch-clamp technique. Use of Ci-VSP allows rapid and reversible control of phosphatase activity through voltage-clamp recordings, which in turn has allowed us to discern the macroscopic closing and opening Kir2 gating kinetics induced by PIP$_2$ de-phosphorylation and re-phosphorylation.

Figure 2C and D shows an acceleration of the gating kinetics of the V223L mutant relative to the WT by activation (left panels) or inhibition (right panels) of Ci-VSP.
PIP$_2$-induced gating mechanism of Kir channels

(kinetics of current inhibition - $\tau_{\text{off}}$ - of V223L: 4.78±0.51s versus WT: 17.73±0.72s; kinetics of current recovery - $\tau_{\text{on}}$ - of V223L: 6.28±0.36s versus WT: 15.72±1.66s). Ci-VSP reduces membrane PIP$_2$ levels when the membrane is depolarized (36). Alternatively, we examined the gating kinetics of activation and deactivation of Kir2.1 WT and its V223L mutant by perfusion and washout of exogenous diC8 PIP$_2$ in inside-out patches. Following patch excision we perfused the patch with a high potassium solution (HK) lacking ATP, which results in PIP$_2$ dephosphorylation and rundown of currents. After complete rundown, diC8 PIP$_2$ was applied at the intracellular side of patches expressing WT or V223L to reactivate the channels. The kinetics of both the rundown ($\tau_{\text{off}}$) (right panels) and reactivation ($\tau_{\text{on}}$) (left panels) by diC8 PIP$_2$ were accelerated by the V223L mutation ($\tau_{\text{off}}$ for WT: 21.97±1.42s and for V223L: 3.44±2.09s; $\tau_{\text{on}}$ for WT: 7.25±1.71s and for V223L: 4.05±0.73s (Fig. S2).

We then tested the effects of the V223M mutation on the PIP$_2$-dependent gating kinetics. The apparent affinity of the WT and V223M channels to diC8 PIP$_2$ were indistinguishable (EC$_{50}$ for WT: 2.75±0.12μM and for V223M: 2.62±0.46μM) (Fig. S3A). V223M also showed similar amplitude currents in TEVC as the WT Kir2.1 (Fig. S3B). Moreover, the kinetics of Ci-VSP-induced changes in currents were also not significantly different for WT versus the V223M mutant ($\tau_{\text{off}}$ for WT: 17.73±0.72s and for V223M: 16.93±0.95s; $\tau_{\text{on}}$ for WT: 15.72±1.66s and for V223M: 12.27±0.61s) (Fig. S3C and D). Thus, V223M, unlike the V223L mutant, displayed similar apparent affinity to PIP$_2$, whole-cell current level and gating kinetics to the WT Kir2.1 channel.

We next asked whether other Kir2 channels showed similar effects as the Kir2.1(V223L) mutant did. Kir2.2 also has a Val (V224) at the analogous position to the Kir2.1(V223) (Fig. S4A). The Kir2.2(V224L) mutant, like the Kir2.1(V223L) mutant, accelerated both $\tau_{\text{off}}$ (left panels) and $\tau_{\text{on}}$ (right panels) ($\tau_{\text{off}}$ for WT: 42.87±7.45s and for V224L: 1.36±0.22s; $\tau_{\text{on}}$ for WT: 176.88±19.42s and for V224L: 13.53±0.43s) (Fig. S4B and C). Unlike Kir2.1, the apparent affinity of the Kir2.2(V224L) mutant for diC8-PIP$_2$ was left shifted (EC$_{50}$ for WT: 6.13±0.43μM and for V224L: 2.46±0.13μM) (Fig. S4D). The WT and the V224L Kir2.2 mutant showed similar whole-cell currents (Fig. S4E).

V223L and V224L but not V223M, also increase the flexibility of the GH loop in the linker between the CD and G loops. In order to explore the mechanism by which the V223L and V224L affected the PIP$_2$-induced gating kinetics, we performed 10ns molecular dynamics (MD) simulations of the cytosolic domains of the WT Kir2.1, Kir2.2 channels and the three mutants (V223L, V223M and V224L) and analyzed the root mean square fluctuation (RMSF), which is a measure of the flexibility of each residue for the WT and mutant channels. Figure 3A shows significant changes in the flexibility of the GH loop (amino acids 270 to 294, which precisely define the boundaries of the GH loop, see Fig. S1) that were induced specifically by the V223L or V224L mutation compared to the WT or the V223M mutant, respectively.

We asked whether this enhanced flexibility of the Val to Leu mutation could be related to its effects on the channel gating kinetics. To address this question, we examined the PIP$_2$-induced gating kinetics of the WT Kir2.1 and V223L mutant channels in intracellular solutions with increased viscosity. The dynamics of a
protein in solution are intimately coupled to the dynamics of the solvent; the fluctuation amplitudes and relaxation rates, i.e. the flexibility of proteins can be reduced by increases in solution viscosity (37-40). Previous studies have shown that increases in solution viscosity affect the moving parts of voltage-dependent Na\(^+\) channels during gating, causing a decrease in the rate of channel activation (41). We increased the viscosity of the intracellular solution by addition of 2M sucrose (42) and measured rundown kinetics of currents from inside-out patches expressing the WT Kir2.1 or the V223L mutant channels. Compared to the normal intracellular solution, the solution with the 2M sucrose showed a non-significant trend of slowing down the time course of rundown in the WT (-sucrose: 24.41±8.40s; + sucrose: 27.70±7.90s), (Fig. 3B). In contrast, the rundown kinetics of the V223L mutant were significantly slowed (- sucrose: 3.77±0.53s; + sucrose: 8.78±0.65s).

The E272G mutant mimics the effect of the V223L mutant. If indeed, V223 were to be allosterically coupled to the G loop (at A306) via the flexible region (at E272), could an increase in the relative flexibility of E272 mimic the V223L effects on the Kir2.1 gating? Gly residues are utilized in protein structures to confer flexibility (e.g. the Gly hinge in the inner helix that forms the permeation pathway) (43,44). We introduced a glycine at E272 and tested whether it increased local flexibility and whether it mimicked the gating effects of V223L. The RMSF analysis of the E272G mutant indeed showed a significant increase in local flexibility (Fig. 4A). The apparent affinity of the E272G mutant to diC8-PIP\(_2\) was slightly left shifted (EC\(_{50}\) values for WT and the E272G mutant were 2.75±0.12μM and 2.14±0.21μM, respectively) (Fig. 4B), while the mutant displayed similar peak currents (Fig. 4C). Interestingly, and consistent with V223L, the E272G mutant accelerated the gating kinetics of both channel inhibition and reactivation by Ci-VSP (\(\tau_{\text{off}}\) for WT: 17.73±0.72s and for E272G: 9.81±0.68s; \(\tau_{\text{on}}\) for WT: 15.72±1.66s and for E272G: 7.13±0.61s) (Fig. 4D-F). A Gly scan of this region (from 266 to 272, Fig. S5A) revealed that Gly mutations at T268 and I269 in the \(\beta\)G strand were not tolerated well (were either functionally dead or displayed very low activity, respectively), while mutations (P266 and I267) on the N-terminal side of the GH loop were without effect. Mutants at the beginning of the flexible GH loop (V270, H271, and E272) showed significant effects on PIP\(_2\)-induced gating (Fig. S5 B-C).

A conserved gating mechanism linking the CD and G loops. In order to gain structural insights of how PIP\(_2\) might induce channel gating via V223 of the CD loop in a manner that involves the GH loop, we performed 100ns free MD simulations on the WT Kir2.2 and V224L, both in the absence (apo) and presence (holo) of PIP\(_2\) using the full-length Kir2.2 crystal structure (13). Table S1 shows salt bridge interactions between PIP\(_2\) and Kir2.2 during a 20ns interval in the second half of the simulations. The simulation showed PIP\(_2\) to interact with residues Q51, R78, R80, K183, K188, K189, R219, and K220 (Fig. 5A and Table S1) in good agreement with a co-crystal structure of Kir2.2 in complex with PIP\(_2\) (17). Comparison of the apo and holo simulations revealed that PIP\(_2\) releases the N terminus from the CD loop by weakening the interactions between R219-D61 and H222-Q51 (fig. 5B, C and fig. 6A, B). The N terminus then switches to interact with the \(\alpha\)G helix by strengthening the
interactions between D292-R44 and E294-R44 (Fig. 5B, C and Fig. 6A, B). The V224L mutant, with an increased flexibility of the GH loop, reduced the inter-subunit interactions of the N-terminus with the CD-loop residues in the apo state (Q51 – H222, and D61 – R219) and the interactions of the N-terminus with the αG helix in the holo state (D292-R44 and E294-R44) compared to the WT (Fig. 6C, D). Thus the mutant decreased overall interactions of both the CD and GH loops with the N-terminus and increased the flexibility of the GH loop, as if interactions with the N-terminus normally constrain the flexibility of the GH loop. To examine the prediction from the simulations of the importance of the D292 interactions, we tested the effects of the D292G mutation on the Kir2.2 gating kinetics. The D292G mutation accelerated the gating kinetics of both channel inhibition and reactivation by CiVSP (\(\tau_{\text{off}}\) for WT: 42.87±7.45s and for D292G: 20.74±2.93s; \(\tau_{\text{on}}\) for WT: 176.88±19.42s and for D292G: 104.82±7.66s) (Fig. 5 D-G). We also made the E294G mutation but that caused a significant reduction in whole-cell currents (from 6.56±0.73 μA for WT to 0.68±0.03 μA for E294G), making it difficult to characterize further.

**DISCUSSION**

**PIP**<sub>2</sub>-induced channel gating is an intrinsically dynamic process, which is difficult to understand by the few available static crystal structures that are missing key transition steps connecting them. Here we apply a combination of computational and electrophysiological approaches to discern the role of important structural elements in the PIP<sub>2</sub>-induced gating of Kir2 channels, focusing on the cytosolic G-loop gate. We started with MD simulations performed on the crystal structure of the WT Kir2.1 cytosolic domains. Following the MD simulation, we identified that the movements of A306 (of the G loop) correlated highly with V223 (of the CD loop) and E272 (of the GH loop).

Mutagenesis of V223 and kinetic electrophysiological measurements showed that V223L but not V223M right-shifted the diC8-PIP<sub>2</sub> dose-response but it increased currents and caused faster gating kinetics, accelerating both the inhibition and recovery rates. The corresponding mutant in Kir2.2, V224L, showed similar kinetic effects to V223L. Additional MD simulations performed on the Kir2.1 (V223L) and Kir2.2 (V224L) showed similar increases in the flexibility of residues corresponding to the GH loop of Kir2 channels (i.e. the 270 to 294 residues of the Kir2.1 or the 265 to 294 residues of the Kir2.2).

A number of crystal structures of KirBac3.1 (21) revealed interactions between the N terminus and the CD loop, forming the “latched” or closed state. In a recent computational study with the Kir3.1 chimera, we revealed that PIP<sub>2</sub> weakens these interactions forcing the latched N terminus to transition to an “unlatched” or open state (45). The fact that the N-terminal interactions underlie the transition of the G-loop gate from the closed to the open state in such different channels as the Kir3.1 chimera and KirBac3.1, suggest that the N-terminal critical role in Kir channel gating has been conserved from prokaryotes to eukaryotes.

Our 100ns-long MD simulations were performed on the Kir2.2 WT or the V224L channel in the presence or absence of PIP<sub>2</sub>. There was excellent agreement between the predicted Kir2.2 interacting residues with PIP<sub>2</sub> (Q51, R78, R80, K183, K188, K189,
R219, and K220) and those reported for Kir2.1 from mutagenesis and electrophysiological measurements (8) (Kir2.2: corresponding residues: Q51, R65, K183, R186, K188, K189, R190, R219, K220, R229, and R313). Similar agreement exists with Kir2.2 residues shown in a recent crystal structure (17) (R65, R78, W79, R80, K183, R186, K188, K189, R190 and R219). Thus, six of the eight residues predicted to interact with PIP₂ were shown to affect PIP₂ sensitivity experimentally (Table S1). Other residues shown experimentally to affect PIP₂ sensitivity could be doing so in an allosteric manner. The interaction network between the CD loop, the N terminus and the loop segment around the αG helix of the GH loop revealed that there were appreciable interactions between the N-terminus and the CD loop (H222-Q51 and R219-D61) in the absence of PIP₂ stabilizing the closed state. PIP₂ weakened these two pairs of interactions, interacting with R219 directly and stabilizing interactions between the N-terminus and the GH loop: D292-R44 and E294-R44. Interestingly D292 and E294 are exactly at the other end of the GH loop from E272 that affected PIP₂-induced gating kinetics. Thus, the N terminus appears to have switched from stabilizing the CD loop in the closed state to stabilizing the αG helical region within the GH loop in the open state.

The V224L mutation not only stabilized the CD-loop and N-terminal interactions with PIP₂ but also weakened all four pairs of interactions between the N-terminus and the CD loop or the GH loop. The weakened H221-Q51 and R219-D61 interactions would make it easier for the N-terminus to switch from the closed to the open state, accelerating the activation kinetics of Kir2 channels. The weakened interactions between the N terminus and αG helical region of the GH loop, allowed for the increased flexibility of the GH loop, ensuring that the channel can easily transition from the open to the closed state, thus accelerating the deactivation kinetics as well. The flexibility of the GH loop (residues from 270 to 294), reflecting the weakened interactions between the N-terminus and the GH loop, showed greater effects on the deactivation kinetics (τoff).

Acknowledgments—We are grateful to Heikki Vaananen and Sophia Gruszecki for oocyte isolation. We thank all members of the Logothetis laboratory for critical feedback on this work at every stage of its development.

REFERENCES


FOOTNOTES
This work is supported by grant HL059949 from NIH to DL, 11145003 from NSFC to HA, 10975045 from NSFC and C2009000029 to YZ, 30730031 from NSFC, 2007CB512100 from the 973 Program and C2009001104 to HZ.

FIGURE LEGENDS

FIGURE 1. MD simulation performed on C terminal domain of Kir2.1 shows highly correlated movements among V223, E272, and A306. A, Two Kir2.2 subunits shown with selectivity filter (SF), Inner Helix gate (or M2 gate), cytosolic G-loop gate indicated. Similarly, the CD and GH loops that regulate the stability of the G-loop gate have been highlighted. B, Correlation of movements among C terminal residues of Kir2.1. V223, E272 and A306 are shown in ▲, ♥ and ♣, respectively. C, Schematic structure of one subunit cytosolic domain, where the Kir2.1 residues, V223, E272 and A306 are shown as small spheres. The pink spheres, in panel A, represent the potassium ions in the ion pathway. In panel A and C, the CD, GH and G loops are highlighted as pink, grey and green, respectively.
FIGURE 2. V223L mutation accelerates the gating kinetics of Kir2.1. A, Dose-response of diC8 PIP$_2$ of Kir2.1 and V223L, showing a right shift in the apparent affinity of PIP$_2$ sensitivity as a result of the mutation. B, Peak currents of Kir2.1 and V223L do not show a decrease in whole-cell current as other mutations do that decrease the apparent channel affinity to PIP$_2$. C, The representative time course of inhibition (PIP$_2$ dephosphorylation induced by Ci-VSP, a 3.7-fold effect) and reactivation (PIP$_2$ rephosphorylation, a 2.5-fold effect) of WT-Kir2.1 and V223L. D, Bars in D are the time constants (mean±SEM of at least six experiments) corresponding to C.

FIGURE 3. Induction of flexibility of the linker between the CD and G-loops is coupled with changes in the gating kinetics of Kir channels. A, RMSF (root mean square fluctuation) analysis of the linker between CD and G-loops of Kir2.1, Kir2.2 and their mutants. The Val to Leu mutations but not the Met mutation cause an increase in the RMSF of Kir2.2 B, Time constants (mean±SEM of at least three experiments) of rundown (due to diC8 PIP$_2$ washout) of WT-Kir2.1 (left) and V223L (right) in the presence and absence of sucrose. Only the Kir2.1(V223L) shows a significant slowing of the rundown kinetics in the presence of sucrose.

FIGURE 4. The Kir2.1(E272G) also accelerates the gating kinetics albeit to a smaller extent than V223L. A, The RMSF of Kir2.1 and E272G in the GH loop, the linker between CD and G-loops. The mutant causes an increase in RMSF relative to the control. B, Dose-responses of diC8 PIP$_2$ on Kir2.1 and the E272G mutant reveal a small leftward shift in apparent affinity caused by the mutation. C, Peak currents of Kir2.1 and the E272G mutant appear comparable. D, The representative time course of current inhibition (PIP$_2$ dephosphorylation induced by Ci-VSP, a 1.8-fold effect) of WT-Kir2.1 and E272G. E, The representative time course of reactivation (PIP$_2$ rephosphorylation, a 2.2-fold effect comparable to the inhibitory effect shown in d) of WT-Kir2.1 and E272G. F, Summary bars (left and right) represent the time constants (mean±SEM of at least six experiments) corresponding to D and E, respectively.

FIGURE 5. Partial crystal structure model of Kir2.2 WT shows detailed interactions between CD-loop, N terminus and the αG helix. A, shows the residues that interact with PIP$_2$ from our 100ns long MD simulations. PIP$_2$ is shown in a ball and stick representation. B and C, show the detailed structures of WT Kir2.1 with (holo) without (apo) PIP$_2$, respectively to underscore changes in the relationship of the N-terminus with the CD and GH loops. D and F, are representative trace currents of Kir2.2 WT and D292G, one of the GH loop residues predicted by our simulations to interact with PIP$_2$. PIP$_2$ dephosphorylation of the D292G mutant induced by Ci-VSP (a 2.1-fold effect) and PIP$_2$ rephosphorylation (a 1.7-fold effect) relative to the control, respectively. E and G, are summary data (mean±SEM of at least six experiments).

FIGURE 6. PIP$_2$ modulates gating of Kir channels by altering interactions between CD-loop, N terminus and the αG helix of the GH loop. A, Effect of PIP$_2$ on averaged hydrophobic (Hp) interactions between R219-D61 and B, Salt Bridge (SB) interactions
between H221-Q51, E292-R44 and E294-R44 in Kir2.2 WT. C, Effect of PIP₂ on averaged hydrophobic (Hp) interactions between R219-D61, and D, Salt Bridge (SB) interactions between H221-Q51, E292-R44 and E294-R44 in Kir2.2 V224L.
A

RMSF (0.1nm)

Kir2.1
V223L
V223M

Residues

Kir2.2
V224L

Residues

B

WT Kir2.1

τ_{off} (s)

HK
HK+Suc

V223L

τ_{off} (s)

HK
HK+Suc
**A**

RMSF (0.1nm) vs. Residues

**B**

Normalized currents vs. diC8 PIP$_2$ (μM)

**C**

Peak currents (μA) vs. Kir2.1 and E272G

**D**

Normalized currents over Time (s)

**E**

Normalized currents over Time (s)

**F**

τ$_{on}$ (s) and τ$_{off}$ (s) for Kir2.1 and E272G
The cytosolic GH-loop regulates the PIP2-induced gating kinetics of Kir2 channels
Hai-Long An, Shou-Qin Lu, Jun-Wei Li, Xuan-Yu Meng, Yong Zhan, Meng Cui, Long Mian, Hai-Lin Zhang and Diomedes E. Logothetis

J. Biol. Chem. published online October 2, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M112.418640

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2012/10/02/M112.418640.DC1

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2012/10/02/jbc.M112.418640.full.html#ref-list-1