Three pairs of weak interactions precisely regulate the G-loop gate of Kir2.1 channel

Junwei Li,1,2 Shaoying Xiao,3 Xiaoxiao Xie,1 Hui Zhou,4 Chunli Pang,1 Shanshan Li,5 Hailin Zhang,6 Diomedes E. Logothetis,7 Yong Zhan,1 and Hailong An1*

1 Key Laboratory of Molecular Biophysics, Hebei Province, Institute of Biophysics, School of Sciences, Hebei University of Technology, Tianjin 300401, China
2 Department of Electrical Engineering and Computer Science, Hebei University of Technology, Langfang 065000, China
3 Department of Urban Planning, School of Architecture and Art Design, Hebei University of Technology, Tianjin 300401, China
4 Department of Mathematics and Physics, North China Electric Power University, Baoding 071003, China
5 Department of Mechatronics Engineering, Hebei University of Technology, Tianjin 300130, China
6 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 050017, China
7 Department of Physiology and Biophysics, School of Medicine, Virginia Commonwealth University, Richmond, Virginia 23298

ABSTRACT

Kir2.1 (also known as IK1) plays key roles in regulation of resting membrane potential and cell excitability. To achieve its physiological roles, Kir2.1 performs a series of conformational transition, named as gating. However, the structural basis of gating is still obscure. Here, we combined site-directed mutation, two-electrode voltage clamp with molecular dynamics simulations and determined that H221 regulates the gating process of Kir2.1 by involving a weak interaction network. Our data show that the H221R mutant accelerates the rundown kinetics and decelerates the reactivation kinetics of Kir2.1. Compared with the WT channel, the H221R mutation strengthens the interaction between the CD- and G-loops (E303-R221) which stabilizes the close state of the G-loop gate and weakens the interactions between C-linker and CD-loop (R221-R189) and the adjacent G-loops (E303-R312) which destabilizes the open state of G-loop gate. Our data indicate that the three pairs of interactions (E303-H221, H221-R189 and E303-R312) precisely regulate the G-loop gate by controlling the conformation of G-loop.

Proteins 2016; 84:1929–1937.
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Key words: Kir channel; targeted molecular dynamics; molecular dynamics; homology model; gating kinetics; weak interaction.

Abbreviations: Ci-VSP, ciona intestinalis voltage-sensitive phosphatase; CTD, cytoplasmic domain; Kir, inward rectifying K+; MD, molecular dynamics; PDB, Protein Data Bank; PIP2, phosphatidylinositol 4,5-bisphosphate; PME, particle-mesh Ewald; POPC, palmitoyloleoyl-phosphatidylcholine; RMSD, root mean square deviation; Targeted MD, targeted molecular dynamics; TEVC, two-electrode voltage-clamp; TMD, transmembrane domain; VMD, visual molecular dynamics

Grant sponsor: Natural Science Fund for Distinguished Young Scholars of Hebei Province of China; Grant numbers: C2015202340, B2014014; Grant sponsor: Fund for Outstanding Talents of Hebei Province of China; Grant number: C201400305; Grant sponsor: Hebei Province Science and Technology Program Self Financing Project; Grant number: 15271704; Grant sponsor: Scientific Innovation Grant for Excellent Young Scientists of Hebei University of Technology; Grant number: 2015010; Grant sponsor: Fund for the Science and Technology Program of Higher Education Institutions of Hebei Province, China; Grant number: QN2016113; Grant sponsor: National Natural Science Fund of Hebei Province; Grant number: C2013202244; Grant sponsor: National Natural Science Fund of China; Grant numbers: 11247010, 11475053, 51505123, 11347017, 31400711.

Yong Zhan and Hai Long An contributed equally to this work.

Author contributions: JL, YZ and HA designed the project and wrote the main manuscript text. JL and HA did most computation and data analysis and prepared all figures. XX, HZ and CP did most of the computational work and data analysis. SX and SL helped with the preparation of some figures. DEL, SL, HZZ, YZ and HA revised the manuscript. All authors reviewed the manuscript.

*Correspondence to: Hai Long An, Institute of Biophysics, School of Sciences, Hebei University of Technology, Tianjin 300401, China. E-mail: hailong_an@hebut.edu.cn

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Received 8 January 2016; Revised 30 June 2016; Accepted 19 September 2016
Published online 3 October 2016 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/prot.25176

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INTRODUCTION

Inward rectifying K⁺ (Kir) channels, intrinsic membrane proteins, selectively permeate potassium ions across cell membranes with primary role in regulating the outward directed K⁺ current. Kir channels regulate many physiological processes, such as pancreatic insulin secretion, vascular tone, cardiomyocytes, heart rate, fluid balance, and K⁺ homeostasis in the body.1–3

The Kir family which includes 16 members has been classified into seven subfamilies (Kir1-7) on the basis of sequence conservation and biophysical properties.4–9 For the past decade, more and more Kir channel structures (the full-length structures and cytoplasmic domain) have been crystallized.10–20 The crystallographic structures reveal that the Kir channels are tetrameric assembly, which has two different regions in series: a transmembrane domain (TMD) and a cytoplasmic domain (CTD). An expansion linker makes the TMD tethered to the CTD, which is named C-linker. The P loop and transmembrane domain (M1-P-M2 motif) form the “transmembrane pore” and the cytoplasmic N- and C-termini (cytoplasmic domain) form “cytoplasmic pore”. The cytoplasmic pore is a common characteristic architecture of Kir channels, which extends the length of the canonical transmembrane pore nearly twice. A long ion permeation pathway outlines both the transmembrane and cytoplasmic pores. Three gates are distributed along the permeation pathway in turn: selective filter gate, the bundle-crossing gate (both in the transmembrane pore) and G-loop gate (in the cytoplasmic pore). At the surface of the cytoplasmic domain a cluster of acidic and hydrophobic residues are found to create a favorable environment for varied modulator (ATP, H⁺, G-protein, PIP₂, and Na⁺ and Mg²⁺)1,21,22 that cause conformational changes of G-loop which is involved in movement of the bundle-crossing gate.8,18,23–27

Experimental data combined with crystal structural studies show that G-loop gate could control the potassium ions flow of Kir channel.12,28

Kir2 subfamily members are well expressed in skeletal muscle, brain, heart and cardiovascular and nervous systems.1–3,7,29 Loss-of-function Kir2.1 mutations, mostly situated in cytoplasmic domain, cause Andersen’s syndrome in human, which is characterized by periodic paralysis, abnormal electrocardiogram and ventricular arrhythmias.12,30,31 It has been suggested that conformational changes of the G-loop are accompanied by transformation of a network of weak interaction in cytoplasmic domain.18,32,33 The E303K and V302M mutations in Kir2.1 channel can change the weak interactions between amino acid of cytoplasmic domain that are able to effect the conformational changes of the G-loop.28,34 However, the key residues near the G-loop of Kir2.1 channel which precisely regulate the gating of G-loop are still far from clear.

Our earlier studies of Kir2.1 channel and mutant demonstrated that weak interactions (E303-H221 and H221-R189) are important for controlling the gating of G-loop.24,33 The previous results show that the residue E303 is essential to maintain the function of Kir2.1 channel.33 To investigate if the H221 is the key residue during the gating, we carried out measurement using to combine site-directed mutagenesis, electrophysiology with molecular dynamics (MD) and targeted MD simulations. Our data show that the H221R mutant accelerates the rundown kinetics, and decelerates reactivation kinetics relative to the WT on account of the change of three pairs of weak interactions.

MATERIALS AND METHODS

Homology modeling

The full-length mouse Kir2.1 channel used as model templates, chain A of 3SPI (chicken Kir2.2 in closed state) and 3SYQ (mouse Kir3.2 in open state), were obtained from the Protein Data Bank (PDB). The homology models were generated using SWISS-MODEL server.35–37 Because of high sequence homology (ca., 77 and 53% sequence identity to cKir2.2 and mKir3.2 respectively), the QMEAN4 score38 was 0.566 and 0.545, respectively and QMEAN Z-score39 was −3.36 and −3.66, respectively. The H221R mutant was generated with software VMD.40 The H221R model was constructed by substituting the His side-chain with the Arg’s. Compared to its template models, the mainchain geometry in experimental models has no change.

Conventional molecular dynamics

For four experimental structures simulation, the structures of wild type (WT) and H221R in closed and open states were embedded in an explicit palmitoyloleylophosphatidylcholine (POPC) bilayer using membrane package, which is a plugin of VMD.40 The two binding sites (S1 and S3) of the selective filter is occupied by K⁺ ion, and the other two (S2 and S4) by water. All molecular dynamics (MD) simulations with explicit solvent and ions were performed in ~0.15 M KCl which was placed randomly to neutralize the system. TIP3P water model41 was employed for the solvent. The solvated systems covered altogether 24–34 ns (including 14-ns equilibration of the lipid and solution with protein released and 10–20 ns production run until reaching equilibrium). Each targeted molecular dynamic was performed on the equilibrated system.

ALL MD simulations with a total of ~140,000 atoms were carried out using the NAMD2 program (http://www.ks.uiuc.edu/Research/namd/)42 and the CHARMM 27 force filed43–45 with NBFIX correction.46–48 The simulations had same characteristics with the reference.24 Constant pressure (1 atm) and constant temperature (310 K) were controlled by Nose’–Hoover–Langevin
piston and Langevin dynamics.\textsuperscript{49,50} Long-range electrostatic interactions, which were calculated using the particle-mesh Ewald (PME),\textsuperscript{51,52} had $120 \times 120 \times 180$ grid points with a grid density of 1 Å\(^3\). Short-range interactions were calculated with a cutoff radius of 12 Å. Smooth switching function was employed at 10 Å and

![Figure 1](image-url)

Figure 1
RMSD variations for four systems (a. Closed and open states of wild type. b. Closed and open states of H221R.) throughout the simulation. RMSDs were calculated based on all C\(\alpha\) atoms of the channel.

![Figure 2](image-url)

Figure 2
(a) The schematic diagram of targeted structure, which is in the open state. (b) The schematic diagram of the initial structure, which is in the closed state. The target residues, to which the targeted force is applied (residues Lys185-Thr192 and PIP\(_2\) binding sites), are colored blue. The G-loop is highlighted as black. (c) The schematic diagram of the final conformation, which is achieved by our last Targeted MD simulation.
distance of pairlist at 13.5 Å. A frame was saved every 4 ps. Simulation analysis and structural diagrams were treated by simulaid and VMD. The Plots were produced by XMGrace program (http://plasma-gate.weizmann.ac.il/Grace). Simulations were performed on a 64-processor Linux cluster.

**Targeted molecular dynamics (targeted MD)**

Targeted MD simulation algorithm\(^5^3, ^5^4\) which was developed by Schlitter et al. was a simple method for producing large-scale conformational change in large proteins within a short simulation time. The conformational change is induced by the steering force which is dependent on the RMSD value. The force is given by the gradient of the potential.

\[
U_{TMD} = k \left[ \text{RMSD}(t) - \text{RMSD}_\ast(t) \right]^2 / 2N
\]

where the force constant is represented by \(k\), \(N\) is the number of atoms which are set as target structure, \(\text{RMSD}(t)\) between the current coordinates and the target structure is computed (after first superimposing the target structure and the initial coordinates), and \(\text{RMSD}_\ast(t)\) evolves linearly from the initial RMSD at the first Targeted MD step to the final RMSD at the last Targeted MD step. \(\text{RMSD}_\ast(t)\) tends to zero is the criterion to end the Targeted MD.\(^5^5\)

**Molecular biology**

All of mouse Kir2.1 channels were subcloned into the pGEMHE expression vector and used as described. H221R mutation was generated using a Quickchange kit (Stratagene). DNA sequencing was used to confirm sequences. As previously described, recombinant wild-type Kir2.1, H221R, and Ci-VSP were used for *Xenopus laevis* oocytes expression.\(^5^6, ^5^7\) cRNA was prepared with T7 RNA polymerase using a kit from Promega. According to the functional expression level, cRNAs of the Kir2.1, H221R and of Ci-VSP were microinjected into *Xenopus* oocytes (0.5–10 ng/oocyte).

**Electrophysiology**

The whole cell currents were recorded on the *Xenopus laevis* oocytes, which were treated 1–2 days after cRNA injected using the conventional two-electrode voltage-clamp (TEVC) with a GeneClamp 500 amplifier (Molecular Devices, CA). Microelectrodes were filled with 3 M KCl dissolved in 1% agarose to prevent the leakage of KCl into the oocytes. Pipettes had a tip resistance of <1 MΩ. The bath solution with a high-potassium (ND96K) contained 96 mM KCl, 1 mM NaCl, 1.8 mM CaCl\(_2\), 1 mM MgCl\(_2\), 5 mM HEPES; the pH was adjusted with KOH to 7.4 at room temperature. A low-potassium solution (ND96) composed of (in mM) 96 NaCl, 1 KCl, 1.8 CaCl\(_2\), 1 MgCl\(_2\), 5 HEPES at a pH of 7.4 with NaOH. ND96 solution was made in some experiments to

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Figure 3

(a) The time course of the interaction between G-loop (blue) and CD-loop (yellow) through intrasubunit E303-H221; (b) Variation of the strengthened interactions between CD-loop (yellow) and C-linker (red) through H221-R189 and between adjacent G-loops through E303-R312 (c) as a function of simulation time. (a–c) are taken the average of H-bond number every 40 ps.
inhibit most of the Kir2.1 currents at $-80 \text{ mV}$. The voltage-pulse protocol for activation of Ci-VSP was carried out on TEVC oocyte recordings: 1s-sweeps contained a 170 ms stepped depolarizations from $-80$ to $+80 \text{ mV}$ and an 830 ms step to $+80 \text{ mV}$. To deactivate Ci-VSP, the holding potential was $-80 \text{ mV}$ on the oocytes. Sweeps were applied till the resulting currents reached steady state. Current amplitudes were elicited by 1s pulses applied to potential ranging from $+80 \text{ mV}$ to $-80 \text{ mV}$. Results are reported as mean ± SEM ($n = \text{number of oocytes}$). Each experiment shown or described was performed on 5–7 oocytes of the same batch for TEVC recording. A minimum of 3–4 batches of oocytes was tested for each experiment shown. Data acquisition and analysis were done with pClamp9.2 (Molecular Devices, CA) and Origin 7.5 (Microcal, MA) software.

RESULTS AND DISCUSSION

Construction, equilibration, and targeted MD simulation

In present study, four full-length structures of Kir2.1 (WT) and Kir2.1 (H221R) channels in closed and open states were constructed using the crystal structures of chicken Kir2.2 (PDB ID: 3SPI) and mouse Kir3.2 (PDB ID: 3SYQ) as the templates. The sequences identities are 77 and 53%, respectively. On the basis of the radius of the pore, we named them closed and open states of Kir2.1, respectively. During 10–20 ns free MD simulations, root mean square deviation (RMSD) values of the four structures are no $>3 \text{ Å}$ which means that these systems reach their equilibration states (Fig. 1). We performed a Targeted MD simulation on the equilibrated closed Kir2.1 WT channel with the targeted structure at the open state [Fig. 2(a,b)]. Figure 2(c) showed the final structure. During this Targeted MD simulation, an external force (force constant: 500 kcal mol$^{-1}$ Å$^{-2}$) was put on the backbone atoms of the PIP$_2$ binding sites (Arg80, Trp81, Arg82, Lys182, Lys185 to Thr192 and Arg218) to make the specific domain reached targeted structure. The simulation showed that three pairs of weak interactions controlled the G-loop gate (Fig. 2, black domain) which is located at the CTD along the pore (Fig. 3). One pair of weak interaction between G- and CD-loop (E303-H221) is decreased, which led the closed G-loop gate to unsteady, made G-loop gate easy to open [Fig. 3(a)]. The other two pairs of weak interactions between the CD-loop and the C-linker (H221-R189) and between the
adjacent G-loops (E303-R312) are increased [Fig. 3(b,c)], which are thought to maintain stability of the G-loop gate in open state.

**H221R mutant affects the gating kinetics of Kir2.1 channel**

To illustrate how the H221 involves the gating of Kir2.1, we designed H221R mutation and measured the gating kinetics of kir2.1 H221R channel, using two-electrode voltage clamp (TEVC) technique. The Ciona intestinalis voltage-sensitive phosphatase (Ci-VSP) was used to control the membrane PIP2 levels in intact cells. Ci-VSP co-expression with WT and H221R mutant channel was activated by voltage protocol in the whole-cell, which was able to achieve rapid and reversible PIP2 depletion. PIP2 de-phosphorylation and re-phosphorylation in turn produced the gating kinetics of Kir2 channel in closing and opening process.

The kinetics of current recovery ($\tau_{on}$) and inhibition ($\tau_{off}$) of H221R through inhibition (left panels) or activation (right panels) of Ci-VSP is four-fold effect of WT ($\tau_{on}$ for H221R: 64.90 ± 6.20s and for WT: 15.73 ± 2.66s; $\tau_{off}$ for H221R: 4.55 ± 0.34s and for WT: 17.73 ± 0.72s) [Fig. 4(a,b)]. The data show that the H221R mutant decelerated the gating kinetics of channel reactivation by PIP2 rephosphorylation, and accelerated by inhibition by PIP2 dephosphorylation compared to WT.

**Three pairs of weak interactions that control the G-loop gate**

To understand how H221 affects the gating kinetics of Kir channel, we performed molecular dynamics (MD) and targeted MD simulation on the H221R Kir2.1 mutant. Compared with the WT channel, the H221R mutation strengthens the interaction between the CD- and G-loops (E303-R221), which stabilizes the close state of the G-loop gate [Fig. 5(a)], and weakens the two pairs of weak interactions between the CD-loop and the C-linker (R221-R189) and between the adjacent G-loops (E303-R312), which could not maintain the G-loop gate in the open state [Fig. 5(b,c)].

To explore the reason why the mutation at Kir2.1 221 position (H221R) led the weak interactions to change, we analyzed the RMSF (root mean square fluctuation) and relative movements between CD- and G-loops. The RMSF is a standard of the flexibility of CD- and G-loop for the WT and H221R mutant. H221R mutation increased the flexibility of the CD-loop (residues 216 to 223) and G-loop (residues 301 to 308) compared to the WT [Fig. 6(a,b)]. From the free MD simulations the Kir2.1 221 position from His to Arg could increase the interaction and correlation between CD- and G-loop [Fig. 6(c,d)], which may cause the enhanced flexibility that could be correlated with its effects on the gating kinetics.60

Figure 5
The three interactions that controls the G-loop gate. (a) The time course of the interaction between G-loop and CD-loop through intrasubunit E303-R221. (b) and (c) show the interaction between CD-loop and C-linker through R221-R189 (b) and between adjacent G-loops through E303-R312 (c). The red and blue line are shown the average of the H-bond number every 40 ps of H221R (red) and WT-Kir2.1 (blue) as a function of simulation time, respectively.
Phosphatidylinositol 4,5-bisphosphate (PIP$_2$) is known to regulate activities of all Kir family, including Kir2.1 channel, through interaction with the positively charged amino acids, which exposed to the surface of interface between CTD and TMD$^{16,56,61,62}$. These binding sites include H53, R67, R82, K182, K185, K187, K188, R189, R218, K219, K228, and R312 (numbered by Kir2.1 channel). The gating of Kir channels is controlled by the inner weak interactions network of the CTD, when PIP$_2$ bound with the binding sites$^{16,24,32}$. Following our previous study, the interactions networks between CD- and G-loop and C-linker play a vital role in the PIP$_2$-induced Kir channels gating.$^{31}$ One pair of weak interactions (E303-H221) between CD- and G-loop may be bound up with the gating of Kir2.1 channel [Fig. 3(a)]. The Kir2.1 E303D mutation disrupted the weak interaction

Figure 6
RMSF (root mean square fluctuation) analysis of the CD-loop (residues 216–223) (a) and G-loop (residues 301–308) (b) of WT-Kir2.1 and H221R. (c) The time course of the interactions between CD-loop and G-loop of H221R (black) and WT-Kir2.1 (green). (d) Correlation of movements among CD-loop and G-loop of WT and H221R.
within the CTD and rendered the dysfunction of Kir2.1. Three pairs of interactions (E303-H221, H221-R189 and E303-R312) precisely regulated the G-loop gate.

**CONCLUSIONS**

To explore the mechanism by which H221 controlled the channel function, we used the combined approaches of computation and electrophysiology. Experimental data show that H221R was able to affect the gating kinetics of Kir2.1 channel (accelerating the inhibition kinetics-τoff and decelerating reactivation kinetics-τon relative to the WT). The simulation data show H221R mutation compared to WT enhanced E303-R221 interaction (between C-linker and CD-loop, adjacent G-loops), which decreased the open time of the G-loop gate. The three pairs of interactions control the gating kinetics of Kir2.1 channel. These results can be helpful for the targeted drug design of Kir channelopathies.

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