E224G Regulation of the PIP_2-Induced Gating Kinetics of Kir2.1 Channels *

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As a member of the inwardly rectifying K^+ channel (Kir) family, Kir2.1 allows K^+ to influx the cell more easily than to efflux, a biophysical phenomenon named inward rectification. The function of Kir2.1 is to set the resting membrane potential and modulate membrane excitability. It has been reported that residue E224 plays a key role in regulating inward rectification. The mutant Kir2.1 (E224G) displays weaker inward rectification than the WT channel. Gating of Kir2.1 depends on the membrane lipid, PIP_2, such that the channel gates are closed in the absence of PIP_2. Here we perform electrophysiological and computational approaches, and demonstrate that E224 also plays an important role in the PIP_2-dependent activation of Kir2.1 in addition to its influence on inward rectification. The E224G mutant takes 4.5 times longer to be activated by PIP_2. To probe the mechanism by which E224G slows the channel opening kinetics, we perform targeted molecular dynamics simulations and find that the mutant weakens the interactions between CD-loop and C-linker (H221-R189) and the adjacent G-loops (R312-E303) which are thought to stabilize the open state of the channel in our previous work. These data provide new insights into the regulation of Kir2.1 channel activity and suggest that a common mechanism may be involved in the distinct biophysical processes, such as inward rectification and PIP_2-induced gating.

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Kir2.1 (IRK1) is an inwardly rectifying K^+ channel, extensively expressed in diverse cell types, including skeletal and cardiac muscle cells, neurons, blood cells and epithelial cells.[1,2] It was first cloned from a mouse macrophage cell line in 1993.[2] Kir2.1 is a tetramer composed of four symmetric subunits (Fig. 1(a)). Each subunit has two membrane-spanning domains with both the C- and N- terminal domains located at the intracellular side of the membrane (Fig. 1(b)).[3] As its name implies, Kir2.1 exhibits pronounced inward rectification, allowing K^+ to move more easily into rather than out of the cell.[1,2,4–8] The mechanism of rectification has been shown to be involved in blocking the outward current by intracellular Mg^{2+}[9,10] or spermine.[8,11–15] Multiple single point mutations[16–21] have been shown to affect the susceptibility to the rectification. Kir2.1 channels have also been shown to possess very high affinity for PIP_2,[6,22–25] a phospholipid localized in the inner leaflet of the membrane. The Kir2.1 channel current diminishes as PIP_2 depleted and its gates are closed in the absence of PIP_2. In other words, Kir2.1 channels are gated by PIP_2.

In 1995, Taglialatela *et al.* reported that the mutation E224G (Fig. 1(b)) reduced rectification induced by Mg^{2+} and spermine[26] showing a much lower sensitivity to Mg^{2+} and/or polyamine.[27,28] They surmised that this effect was due to the weakening of an electrostatic interaction between E224 and R228 or R260 in the mutant. This electrostatic interaction caused the 'pore' in the bundle crossing region to be narrower such that spermine could block the narrowed 'pore' more easily. Thus the mutant reversed this effect and a larger outward current emerged with weaker rectification.[21]

The direct activation of Kir2.1 by PIP_2 was first reported by Huang *et al.*[22] Many residues, such as H53, R67, K185, P186, K187, K188, R189, R218, K219, L222, R228, and R312 have been reported to affect the gating of Kir2.1 induced by PIP_2.[29–31] By combining molecular dynamics (MD) with targeted MD simulations, we previously studied the conformation transition from the closed state to the open state and identified seven pairs of H-bonds that are thought to stabilize the channel in both closed and open states.[32]

Here we investigate the effect of E224G on the gating of Kir2.1 induced by PIP_2. To measure the gating kinetics, we co-expressed in HEK293T cells WT

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or mutant Kir2.1 with C.intestinalis voltage-sensor-containing phosphatase (Ci-VSP) which is regulated by membrane potential[33–38] to control the level of PIP_2.[34,39] We found that the E224G mutation not only weakens the inward rectification but also delays the opening gating kinetics of Kir2.1 channels. The time constants of the opening kinetics for the WT and the mutant E224G channels were 73.5 s and 333.2 s, respectively. In other words, E224 is involved in the PIP_2-dependent gating and inward rectification of Kir2.1, simultaneously. Targeted MD simulation results showed that the E224G mutant weakens the interactions between CD-loop and C-linker (H221-R189) and the adjacent G-loops (R312-E303) that play a very important role in stabilizing the open state of Kir2.1 channel.

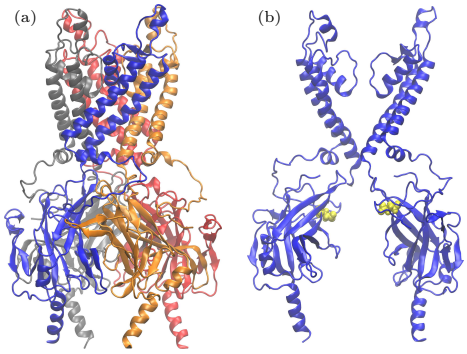

Fig. 1. The structure of Kir2.1 channel. (a) The full-length tetrameric structure of Kir2.1 channel. Each subunit of the tetramer is highlighted in different colors. (b) The two opposite subunits and the location of residue E224 (yellow balls). E224 is located in the cytoplasmic domain internal to the M2 segment in the inner vestibule of the Kir2.1 channel pore.

Firstly, we examined the effect of E224G on the inward rectifying of the Kir2.1 channel using the patch clamp technique. In our experiment, we chose HEK293T cells which were cultured in DMEM supplemented with 10% fetal bovine serum at 37℃ in a humidified atmosphere containing 5% CO_2. One day before transfection, cells were seeded on coverslips (0.13–0.17 mm thick) in 24-well plates. About 12 h later, transient transfection was performed before experiments were run, using the X-tremeGENE HP DNA Transfection Reagent (Roche, Mannheim, Germany). There were 500 ng Kir2.1 plasmid DNA, 1.5 μL X-tremeGENE HP and 50 μL Opti-MEM®I (Life, USA) in every well. Lastly, cells were transferred to an incubator at 37℃ in a humidified atmosphere containing 5% CO_2. The cells 24 hours later were transferred to the patch clamp (HEKA, Lambrecht, Germany) for recording. The pipette solution and the bath solution for the excised inside-out modes of the patch-clamp technique contained in mM: KCl, 116.88; EDTA, 2; KH_2PO_4, 2.83; K_2HPO_4, 7.17 (adjusted to pH7.4 with KOH). The electrodes were made from borosilicate glass capillaries (Vital Sense Scientific Instruments Co., Ltd. Wuhan, China) and were pulled on a Sutter P-97 puller (Sutter Instrument CO. USA) with pipette resistances between 1.5 and 2.5 MΩ. Currents from wild-type and E224G mutant Kir2.1 channel were attained using the excised inside-out patch mode (Fig. 2(a)). The wild-type Kir2.1 channel showed very strong inward rectification. The E224G mutant channel did not alter the inward currents but enlarged the outward currents consistent with the previous reports.[21,26–28] The ratios of the current at +50 mV over that at −80 mV were 0.055±0.004 and 0.35±0.03 for WT and E224G, respectively (Fig. 2(b)). That is to say, the rectification of E224G mutant was reduced 6.4 times compared with that of the WT.

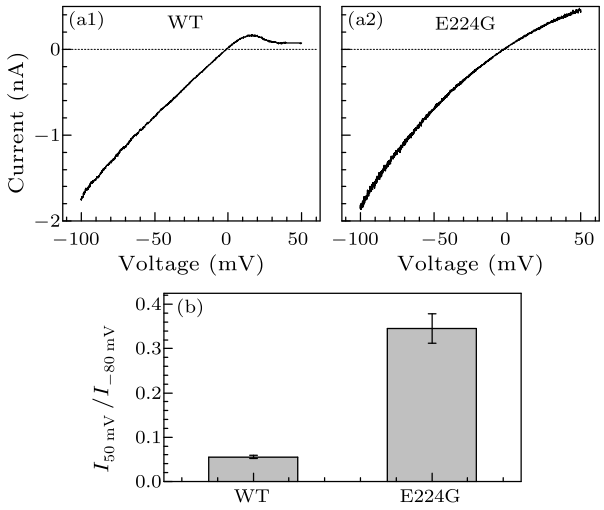

Fig. 2. Rectification in WT and V224G of Kir2.1. (a) WT and E224G currents recorded from transfected HEK293T cells under excised inside-out patch clamp by applying ramp pulses of 800 ms from −100 to +50 mV. (b) Bars are the ratios of the current at +50 mV over −80 mV. The mean and standard deviations of each group are 0.055±0.004 ($n = 4$) for WT and 0.35±0.03 ($n = 5$) for E224G, respectively.

Next we moved to measure the gating kinetics of Kir2.1 by Ci-VSP. The activation of Ci-VSP could deplete PIP_2 and the inhibition of Ci-VSP would permit resynthesis of PIP_2. The Kir2.1 and Ci-VSP were cotransfected to HEK293T cells. There were 250 ng Kir2.1 plasmid DNA, 250 ng Ci-VSP plasmid DNA, 1.5 μL X-tremeGENE HP and 50 μL Opti-MEM®I in every well. The Kir2.1 currents recorded under the on-cell mode decreased gradually upon depletion of PIP_2 by activation of Ci-VSP through membrane depolarization (Fig. 3(a)). The pipette solution and the bath solution are the same as mentioned above. The voltage protocols (top) were 50 ms at 0 mV, 50 ms at −80 mV and 900 ms at +100 mV (Fig. 3(a) top). The inward currents recorded at −80 mV decreased step-by-step (Fig. 3(a) bottom). We refer to the channel

closing time as the time taken for the inward currents to go from maximal to zero. The time courses of the normalized inhibited currents are shown in Fig. 3(b). The plots were fitted by an exponential decay function with $\tau_{\rm off}$ being the time constant and representing the closing time. Here $\tau_{\rm off}$ values were 1.58±0.11 s and 0.79±0.13 s for WT and E224, respectively (Fig. 3(c)).

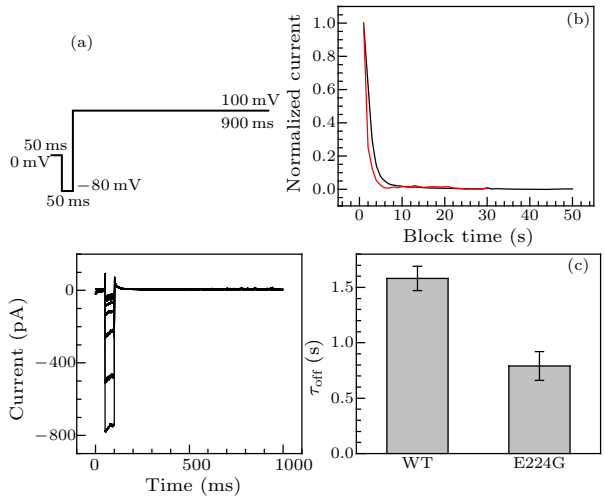

Fig. 3. The inhibitory time course of WT and E224G of Kir2.1. (a) Voltage protocols (top): 50 ms at 0 mV, 50 ms at −80 mV and 900 ms at +100 mV yielding a gradually decreased current in the on-cell model at −80 mV (bottom). (b) Temporal course of the current amplitude at −80 mV for WT (black) and E224G (red). (c) Bars represent the time constants ($n > 5$) corresponding to (b) which were fitted by single exponential equations 1.58±0.11 s for WT and 0.79± 0.13 s for E224G, respectively.

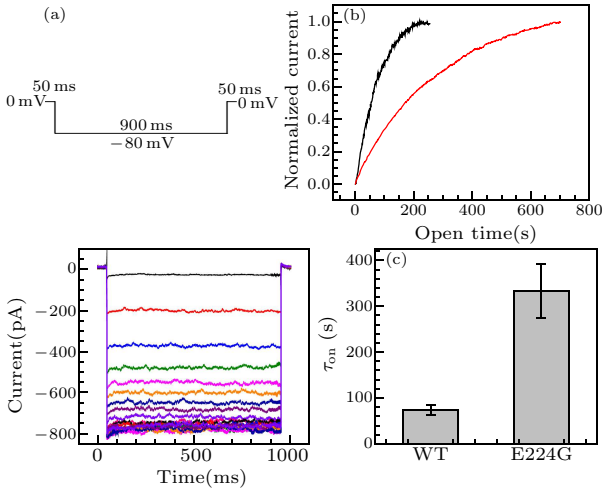

Fig. 4. The opening time course of WT and E224G of Kir2.1. (a) Voltage protocols (top) 50 ms at 0 mV, 900 ms at −80 mV and 50 ms at 0 mV yielding a gradually increased current in the on-cell patch clamp mode at −80 mV (bottom). (b) Temporal courses of the current amplitude at −80 mV for WT (black) and E224G (red). (c) Bars represent the time constants ($n > 5$) corresponding to (b) which were fitted by single exponential equations 74±11 s for WT and 333±58 s for E224G, respectively.

When Ci-VSP is deactivated by hyperpolarization, PIP_2 will be resynthesized in the cell, the currents of Kir2.1 are recovered gradually. To record the process, the voltage protocol was 50 ms at 0 mV, 900 ms at −80 mV and then 50 ms at 0 mV (Fig. 4(a) top). Ci-VSP was deactivated at −80 mV and the currents increased gradually as PIP_2 was re-synthesized in HEK293T (Fig. 4(a) bottom). The time course of normalized currents is shown in Fig. 4(b) for WT and E224G mutant, respectively. The plots were fitted with an exponential function with the time constant $\tau_{\rm on}$ representing the opening time. Here $\tau_{\rm on}$ were 74± 11 s and 333±58 s for WT and E224G, respectively (Fig. 4(c)). The time taken for the E224G channel to re-activate was 4.5 times longer than the time it took for WT re-activation. Thus the E224G mutation greatly delayed the channel activation kinetics compared with the WT control.

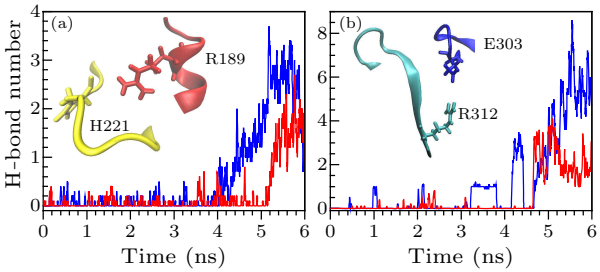

Fig. 5. The weaker interactions that control the G-loop gate. The red and blue lines show the average of the H-bond number every 40 ps for E224G (red) and WT-Kir2.1 (blue) as a function of simulation time, respectively. The time courses of the interaction between the CD-loop and the C-linker represented by the H221-R189 pair (a) and between adjacent G-loops through E303-R312 pair (b).

To understand the molecular mechanism by which the E224G regulates the gating dynamics, we perform targeted MD on the WT and E224G mutant from the activated to the open channel state. The two homology models of Kir2.1 channel based on the activated state of the chicken Kir2.2 (PDB code: 3SPI) and the open state of the mouse Kir3.2 (PDB code: 3SYQ) were achieved by using the SWISS-MODEL server. MD simulations are performed with the NAMD2 program and the structures reach their equilibration states within 20 ns. To obtain the transition from the activated state to open state we run the targeted MD and a steering force with a force constant of 500 kcal/mol/Å^2 was applied to the C-linker and the PIP_2 binding sites. More details can be found in the previous work.[32] Our data show that the E224G mutant weakens the two interactions between CD-loop and C-linker (H221-R189) and between adjacent G-loops (R312-E303), which are thought to stabilize the open state (Fig. 5).

The Kir2.1 channel exhibits Mg^{2+} and spermine-dependent inward rectification and PIP_2-dependent gating[40] which are thought to be two distinct biological processes. Here we combined experimental with computational methods and demonstrated that E224

involves both inward rectification and the gating of Kir2.1. Mutation at this residue weakens the affinity of the channel to Mg^{2+} or spermine then enlarges the outward currents through the channel. Meanwhile, the mutant prolongs the activation process of Kir2.1 by weakening the interaction between H221-R189 and R312-E303, which are thought to stabilize the open state. Our data indicate that a common mechanism may be involved in the distinct biophysical processes, such as inward rectification and PIP_2-dependent gating.

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