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Addendum

Control of pH and PIP_2 Gating in Heteromeric Kir4.1/Kir5.1 Channels by H-Bonding at the Helix-Bundle Crossing

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H-Bonding at the Helix-Bundle Crossing Controls Gating in Kir Potassium Channels

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ABSTRACT

Inhibition by intracellular H^+ (pH gating) and activation by phosphoinositides such as PIP_2 (PIP_2-gating) are key regulatory mechanisms in the physiology of inwardly-rectifying potassium (Kir) channels. Our recent findings suggest that PIP_2 gating and pH gating are controlled by an intra-subunit H-bond at the helix-bundle crossing between a lysine in TM1 and a backbone carbonyl group in TM2. This interaction only occurs in the closed state and channel opening requires this H-bond to be broken, thereby influencing the kinetics of PIP_2 and pH gating in Kir channels. In this addendum, we explore the role of H-bonding in heteromeric Kir4.1/Kir5.1 channels. Kir5.1 subunits do not possess a TM1 lysine. However, homology modelling and molecular dynamics simulations demonstrate that the TM1 lysine in Kir4.1 is capable of H-bonding at the helix-bundle crossing. Consistent with this, the rates of pH and PIP_2 gating in Kir4.1/Kir5.1 channels (two H-bonds) were intermediate between those of wild-type homomeric Kir4.1 (four H-bonds) and Kir4.1(K67M) channels (no H-bonds) suggesting that the number of H-bonds in the tetrameric channel complex determines the gating kinetics. Furthermore, in heteromeric Kir4.1(K67M)/Kir5.1 channels, where the two remaining H-bonds are disrupted, we found that the gating kinetics were similar to Kir4.1(K67M) homomeric channels despite the fact that these two channels differ considerably in their PIP_2 affinities. This indicates that Kir channel PIP_2 affinity has little impact on either the PIP_2 or pH gating kinetics.

INTRODUCTION

Inwardly-rectifying K^+ (Kir) channels belong to a class of K^+ channels that are expressed in almost every cell type and which regulate a variety of processes including membrane excitability, heart rate, vascular tone, insulin release and salt flow across epithelia. The activity of Kir channels is controlled by diverse cellular modulators including phosphoinositides, G-proteins, intracellular Na^+, pH and ATP.[1,2] But whereas some of these factors only regulate specific Kir channel subtypes (e.g., G-proteins and Kir3.x channels, ATP and Kir6.x channels), pH and PIP_2 are more promiscuous, regulating the activity of almost all Kir channels. Phosphoinositides (especially PIP_2) can be considered indispensable cofactors for Kir channels as no activity is normally observed in their absence. Thus, not surprisingly, mutations that interfere with PIP_2 binding result in disease states such as Andersen's Syndrome, Bartter's Syndrome and hyperinsulinaemias.[3-5]

All Kir channels are inhibited by low intracellular pH, however, their pH sensitivity differs markedly. A key determinant of this pH sensitivity is a lysine residue in the first transmembrane domain (TM1) at the helix-bundle crossing. Kir channels with a lysine at this position (Kir1.1, Kir4.1, Kir4.2 and Kir4.1/Kir5.1) show high pH sensitivity. By contrast, channels lacking a lysine at this position (e.g., Kir 2.1, Kir3.x and Kir6.2) are less pH sensitive, but their pH sensitivity can be increased if a lysine is introduced, indicating that the pH gating machinery is conserved in all Kir channel subunits. Originally it was thought that this lysine was the pH-sensor.[6-8] However, recent work has shown that it is not the actual titratable H^+-sensor, but instead a critical component of the gating machinery.[9,10] Thus, the molecular identity of the H^+-sensor remains unknown, and may involve multiple titratable residues in the cytoplasmic domains.[11]

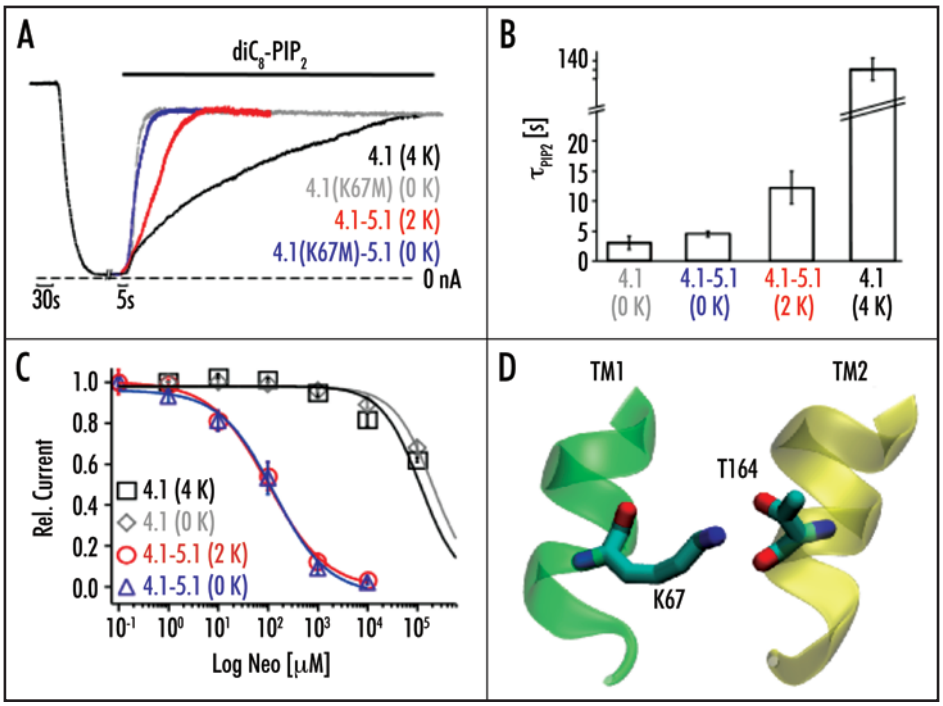

Figure 1. H-bonding in Kir4.1 controls the speed of PIP_2 activation in heteromeric Kir4.1/Kir5.1 channels. Kir channels were expressed in *Xenopus* oocytes and currents measured in excised inside-out patches (described in Rapedius et al.[12]). (A) Time course for Kir4.1 channel rundown (induced by polylysine, only Kir4.1-WT shown) and reactivation by 30 μM diC8-PIP_2 for Kir4.1 (4 K) ((X K) denotes the number of TM1 lysines); Kir4.1-K67M (0 K), Kir4.1/Kir5.1 (2 K) and Kir4.1-K67M/Kir5.1 (0 K) channels. (B) Bars represent tau_{PIP2} (mean ± SEM) for diC8-PIP_2 activation determined from monoexponential fits to the diC8-PIP_2 activation time course (data for Kir4.1 and Kir4.1(K67M) were from Rapedius et al.[12] and shown here for better comparison) (C) Dose-response curves for neomycin inhibition fitted to a standard Hill function with a IC_{50} values and Hill coefficients as follows: 120 mM ± 30 mM and 1.15 (constrained) for Kir4.1 channels (n = 6), 210 mM ± 30 mM and 1.15 (constrained) for Kir4.1-K67M channels (n = 7), 110 ± 50 μM and 1.1 ± 0.4 for Kir4.1/Kir5.1 channels (n = 5) and 130 ± 40 μM and 1.2 ± 0.4 for Kir4.1-K67M/Kir5.1 channels (n = 5). (D) Side view of one Kir4.1 subunit of our Kir4.1/Kir5.1 model depicting parts of TM1 (in green) and TM2 (in yellow) at the helix bundle crossing and showing the proximity and orientation of the side chains of K67 and T164; oxygen atoms in red and nitrogen atoms in blue; note: during our MD simulation the ε-nitrogen of K67 formed H-bonds with both the backbone carbonyl and side-chain oxygens of Thr-164.

H-BONDING AT THE HELIX-BUNDLE CROSSING CONTROLS PH AND PIP_2 GATING IN KIR CHANNELS

In a recent study of Kir channel gating we uncovered a critical role of this TM1 lysine in both the PIP_2 and pH gating mechanisms.[12] We found that channel activation, induced either by a rapid increase in membrane bound diC8-PIP_2, or an increase in intracellular pH following H^+ inhibition, was dramatically affected by the type of residue at this TM1 position. For example, wild-type Kir1.1 channels with a lysine at this position (K80) activated very slowly, whereas substitution of K80 with any residue other than glutamine resulted in rapid PIP_2 and pH gating kinetics. Similar results were found with Kir4.1 which also has a lysine at this position (K67).

Homology modelling and molecular dynamics (MD) simulations of a Kir1.1 closed state structure indicated that the $-NH_3^+$ group of the lysine (K80) and the $-NH_2$ of glutamine (K80Q) residues were able to form a hydrogen bond with the backbone carbonyl group of A177 in TM2 at the helix bundle crossing, but that all other substitutions at K80 either lacked an appropriate H-donor, or were too far from A177 to form an H-bond.[12] Current models indicate that Kir channel activation would require this H-bond to be ruptured whilst the TM segments splay apart during opening,[13,14] thus providing a structural explanation for the striking correlation between the ability of these residues to H-bond and the pH and PIP_2 gating kinetics. It also provides a straightforward explanation as to why a lysine at this TM1 position enhances pH sensitivity in Kir channels as H-bonding would stabilize the closed state induced by channel protonation. We were also able to demonstrate that mutations in TM1 could alter this PIP_2 activation rate without affecting PIP_2 affinity, thus distinguishing between the PIP_2 binding step and the subsequent conformational steps which take place during channel activation.[12]

In this addendum to our previous study[12] we investigate the role of TM1-TM2 H-bonding in the heteromeric Kir4.1/Kir5.1 channel and find that our results support a general model where Kir channel gating kinetics are influenced by the strength of TM1-TM2 H-bonding at the helix-bundle crossing.

RESULTS AND DISCUSSION

Homology modeling shows H-bonding in heteromeric Kir4.1/Kir5.1 channels. In our previous studies we used a homology model of Kir1.1 based upon the transmembrane domain structures of KirBac1.1 and KirBac3.1, and the intracellular domains of Kir2.1 and Kir3.1.[9,12] However, a recently published high-resolution crystal structure of a chimeric Kir3.1/KirBac1.3 channel[15] presents an opportunity to validate our Kir1.1 homology model. Comparison of the tetramers and the monomers of these structures produced RMSD values of 2.5Å and 2.3Å, respectively. However, when the Kir1.1 model was broken down into its modular parts and the N-terminus, the TM domains (excluding a loop that is not present in both proteins) and the C-terminus were compared we found that the RMSD values dropped to 1.2Å, 1.0Å and 1.7Å respectively. This indicates that the individual domains, especially the TM domains, are very similar and validates the approach taken to construct our original homology model.

We therefore decided to use a similar approach[9] to construct a homology model of a heteromeric Kir4.1/Kir5.1 channel and to explore the role of H-bonding in these channels. The Kir4.1 subunit has a lysine at the TM1 position (K67), whereas Kir5.1 has a methionine (M73) at the corresponding position. During a 5 ns MD simulation, we found that the side chain ε-nitrogen of Lys-67 in Kir4.1 not only formed an intra-subunit H-bond with the backbone carbonyl of Thr-164, but also with the side-chain oxygen of Thr-164. Figure 1D depicts the location and orientation of Lys-67

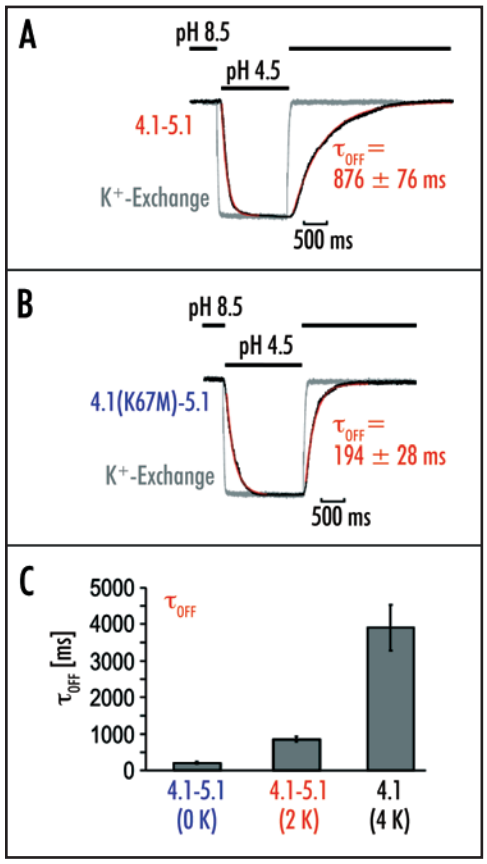

Figure 2. H-bonding in Kir4.1 controls the pH gating kinetics in heteromeric Kir4.1/Kir5.1 channels. (A) Time course of pH gating in Kir4.1/Kir5.1 and (B) in Kir4.1-K67M/Kir5.1 channels induced by rapid changes in the intracellular pH established by a fast piezo-driven application system (described in Rapedius et al.[12]); time course of respective Kir currents upon K^+ exchange (replacement with Na^+ measured at +40 mV) are shown in grey (tau values for on and off rates were between 30 to 60 ms) and superimposed on the pH gating time course obtained in the same patch. (C) The time course of recovery from H^+ inhibition obtained from experiments as shown in (A and B) were fitted with monoexponetial functions and the respective tau values (τ_{off}) were plotted for the indicated WT and mutant channels (data for Kir4.1 were from Rapedius et al.[12] and shown here for better comparison).

and shows the proximity of the ε-nitrogen to both the backbone carbonyl and side-chain oxygens of Thr-164. The ε-nitrogen and these oxygens approached as close as 2.6Å and 2.7 Å, respectively, suggesting capability for H-bonding. Indeed, during the MD simulation of our closed-state model we observed hydrogen bonds forming between K67 and T164 for 53% (Kir4.1 subunit A) and 21% (Kir4.1 subunit B) of the time. This degree of hydrogen bonding agrees well with our previous modelling of Kir1.1.[12] As expected, no hydrogen bonds were observed between the equivalent residues (M73 and A167) in either Kir5.1 monomer.

H-bonding in Kir4.1 controls PIP_2 activation in Kir4.1/Kir5.1 channels. To functionally determine the role of H-bonding in Kir4.1/Kir5.1 we measured their PIP_2 and pH gating kinetics in comparison to homomeric Kir4.1. Figure 1A shows the time course of channel activation upon fast application of 30 μM diC8-PIP_2 for Kir4.1/Kir5.1, Kir4.1(K67M)/Kir5.1, Kir4.1 and Kir4.1(K67M) channels. The speed of PIP_2 activation for Kir4.1/Kir5.1 (two H-bonds) with corresponding tau_{PIP2} of 12 ± 3 s was intermediate between wild-type homomeric Kir4.1 (four H-bonds; tau_{PIP2} = 119 ± 3 s, Rapedius et al.[12]) and Kir4.1 (K67M) (no H-bonds; tau_{PIP2} = 3 ± 1 s, Rapedius et al.[12]). Furthermore, disruption of the remaining two H-bonds in Kir4.1(K67M)/Kir5.1 channels resulted in a PIP_2 activation with a tau_{PIP2} of 4 ± 0.5 s similar to Kir4.1(K67M) channels, which also lack H-bonding at the helix-bundle crossing (Fig. 1B).

We next determined the apparent PIP_2 affinity of these channels using neomycin inhibition as an assay[12,16] (Fig. 1C). We found that the IC_{50} for neomycin inhibition was about 1000 fold higher in Kir4.1 (120 ± 30 mM) or Kir4.1(K67M) (210 ± 30 mM) channels compared to Kir4.1/Kir5.1 (110 ± 50 μM) or Kir4.1(K67M)/Kir5.1 (130 ± 40 μM) channels. This demonstrates that the degree of H-bonding in any given channel does not influence the apparent PIP_2 affinity, similar to what was observed for Kir1.1 channels.[12] Furthermore, it demonstrates that the Kir5.1 subunit markedly reduces the PIP_2 affinity in heteromeric Kir4.1/Kir5.1 channels consistent with previous work[17] and clearly indicates that the PIP_2 affinity itself does not determine the PIP_2 activation rate. This is in agreement with our proposed hypothesis that it is not the PIP_2 binding step itself, but a subsequent conformational step (e.g., at the helix bundle crossing) that determines the rate of the PIP_2 gating kinetics.[12]

H-bonding in Kir4.1 determines pH gating in Kir4.1/Kir5.1 channels. We determined the $pH_{0.5}$ value for Kir4.1/Kir5.1 to be 7.5 ± 0.1, for Kir4.1(K67M)/Kir5.1 to be 6.5 ± 0.1, for Kir4.1 to be 6.0 ± 0.1 and for Kir4.1(K67M) to be 4.3 ± 0.1 (data not shown). This is in agreement with previous studies[18,19] and shows that the TM1 lysine determines the pH sensitivity within any given channel complex, although the mechanism by which Kir5.1 enhances the pH sensitivity of Kir4.1/Kir5.1 channels (compared to homomeric Kir4.1) is clearly not related to the degree of TM1-TM2 H-bonding.

Figure 2A and B shows the time course of H^+ inhibition and recovery upon a rapid pH jump from pH 8.5 to 4.5 for Kir.4.1/Kir5.1 and Kir4.1(K67M)/Kir5.1 channels. The inhibition and recovery kinetics displayed monoexponential behavior. The tau values for pH inhibition in these Kir channel were similar (480 ± 160 ms for Kir4.1/Kir5.1 and 620 ± 70 ms for Kir4.1(K67M)/Kir5.1) and also similar to Kir4.1 channels (420 ± 60 ms, Rapedius et al[12]). This is consistent with the concept that H-bonding does not affect the open to closed channel transition.[12] However, the speed of recovery from H^+ inhibition was clearly related to the number of H-bonds with tau values of 190 ± 30 ms for Kir4.1(K67M)/Kir5.1 (no H-bonds), 850 ± 60 ms for Kir4.1/Kir5.1 (2 H-bonds) and 3900 ± 600 ms for Kir4.1 (four H-bonds, Rapedius et al[12]) channels (Fig. 2A–C). (note: The pH gating of Kir4.1(K67M) could not be determined because there was little inhibition even at pH 4.5, see Rapedius et al.[12]). We conclude that, similar to PIP_2 gating, the number of H-bonds determines the rate of channel opening for pH gating in Kir4.1/Kir5.1 channels.

CONCLUDING REMARKS

Interestingly, although the absolute rates for PIP_2 activation and recovery from H^+ inhibition in Kir4.1 channels differ considerably (i.e., a tau value of 4 s for pH gating and 119 s for PIP_2 gating), the fold change in rates caused by the disruption of H-bonding (cf. Kir4.1(K67M)/Kir5.1 and Kir4.1) are similar, i.e., 26-fold for PIP_2 gating and 20-fold for pH gating (note: we cannot directly

use Kir4.1(K67M) for this comparison because of the weak pH inhibition). This would be consistent with a model in which the energy required to break the H-bonds upon channel opening is similar for both PIP_2 and pH gating. However, to reach definitive conclusions about the relationship between H-bonding stoichiometry, gating kinetics and energetics (e.g., the contribution of a single H-Bond) will require future studies using Kir channels with fixed H-bonding stoichiometries.

The perspective by Guy-David and Reuveny[20] which accompanies our previous work suggested that the ability to modulate Kir channel subunit/PIP_2 interactions might be the common and final point that underlies the regulation of Kir channels by such diverse modulators as G proteins, intracellular Na^+, phosphorylation and also pH inhibition. In general we share this view, but believe this idea may prove somewhat misleading with respect to pH inhibition. This is because it might imply that channel protonation causes the unbinding of PIP_2 and that this dissociation is what results in channel closure. However, the rates of PIP_2 and pH gating kinetics do not support this view because the rate of PIP_2 activation is much slower (e.g., about 30-fold in Kir4.1 channels) than the recovery rate from H^+ inhibition. If H^+ inhibition were to promote the dissociation of PIP_2 then the rebinding of PIP_2 (i.e., the PIP_2 activation rate) should govern the time course of recovery from pH inhibition, which is clearly not the case. Furthermore, we report here that Kir4.1 and Kir4.1/Kir5.1 channels differ markedly in their PIP_2 affinity yet their rates of pH-gating are very similar. These findings are more consistent with a model in which H^+ inhibition causes the channel to close with PIP_2 still bound. This does not imply that channel protonation cannot change PIP_2 affinity and vice versa. Indeed, in general Kir channels with a lower PIP_2 affinity show a higher pH sensitivity indicating a possible relationship between the PIP_2-channel interaction and pH sensitivity,[2] but the mechanistic basis of this remains elusive.

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