

Identification of a Heteromeric Interaction That Influences the Rectification, Gating, and pH Sensitivity of Kir4.1/Kir5.1 Potassium Channels*

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Heteromultimerization between different potassium channel subunits can generate channels with novel functional properties and thus contributes to the rich functional diversity of this gene family. The inwardly rectifying potassium channel subunit Kir5.1 exhibits highly selective heteromultimerization with Kir4.1 to generate heteromeric Kir4.1/Kir5.1 channels with unique rectification and kinetic properties. These novel channels are also inhibited by intracellular pH within the physiological range and are thought to play a key role in linking K⁺ and H⁺ homeostasis by the kidney. However, the mechanisms that control heteromeric K⁺ channel assembly and the structural elements that generate their unique functional properties are poorly understood. In this study we identify residues at an inter-subunit interface between the cytoplasmic domains of Kir5.1 and Kir4.1 that influence the novel rectification and gating properties of heteromeric Kir4.1/Kir5.1 channels and that also contribute to their pH sensitivity. Furthermore, this interaction presents a structural mechanism for the functional coupling of these properties and explains how specific heteromeric interactions can contribute to the novel functional properties observed in heteromeric Kir channels. The highly conserved nature of this structural association between Kir subunits also has implications for understanding the general mechanisms of Kir channel gating and their regulation by intracellular pH.

Inwardly rectifying potassium (Kir) channels are not only important for the regulation of the resting membrane potential and cellular electrical activity but are also key regulators of K⁺ transport processes throughout the body (1, 2). One such example of their critical role in K⁺ transport and homeostasis is in the polarized epithelia of the renal tubules. Some 95% of dietary K⁺ absorbed by the intestine is excreted via the kidney and thus control of this process is one of the key mechanisms of K⁺ homeostasis. Kir channels in the apical membrane are responsible for the secretion of excess K⁺, whereas in the basolateral membrane they maintain the negative membrane

potential and allow K⁺ to recycle via the Na⁺/K⁺-ATPase powering most of the transepithelial solute and fluid transport (3).

The apical secretory K⁺ channel in the distal nephron of the kidney is Kir1.1 (ROMK1) (3), whereas one of the basolateral Kir channels has recently been identified as a heteromeric Kir4.0/Kir5.1 channel (4). A key property of these Kir1.1, Kir4.0, and heteromeric Kir4.0/Kir5.1 channels is their inhibition by H⁺ within the physiological range, thus making them highly sensitive linkers between K⁺ and H⁺ homeostasis (5–10). This functional linkage allows coordinate regulation of both apical and basolateral K⁺ channels, as well as providing a mechanism to prevent excessive loss of K⁺ during metabolic acidosis (3).

The mechanism by which these channels sense intracellular pH is unclear, but it is thought to require the presence of a lysine residue in a particular position within the first trans-membrane domain (Lys-80 in Kir1.1 and Lys-67 in Kir4.1). It has been proposed that this lysine residue exhibits anomalous titration and therefore functions directly as the H⁺ sensor (11, 12). Heteromeric Kir4.1/Kir5.1 channels are much more sensitive to intracellular pH than Kir4.1 homomeric channels (Kir4.1 pK_a = 6.1 compared with pK_a = 7.4 for Kir4.1/Kir5.1) (10). However, Kir5.1 does not possess a lysine residue at this equivalent position (Met-73) and the pH sensitivity of the heteromeric Kir4.1/Kir5.1 channels is principally determined by the Kir4.1 subunit. Thus, heteromultimerization with Kir5.1 somehow modulates the "intrinsic pH sensor" within Kir4.1 and this must involve some form of specific heteromeric interaction between these subunits (10).

The ability of Kir subunits to heteromultimerize with Kir5.1 is highly restricted, only Kir4.1 and Kir4.2 physically associate with Kir5.1 and we have recently shown that a small domain within the C terminus of Kir4.1 contributes to the specificity of this interaction (13). Highly selective heteromultimerization is important because individual cells express many different Kir subunits. Thus, for these cells to maintain distinct subpopulations of both homo- and heteromeric Kir channels it is essential that mechanisms exist to prevent random or promiscuous interactions.

Heteromultimerization between Kir4.1 and Kir5.1 also produces an increase in single-channel conductance, an increase in rectification, and very slow time-dependent activation at hyperpolarizing potentials compared with homomeric Kir4.1 (14). This time-dependent activation is one of the most characteristic features of Kir4.1/Kir5.1 heteromeric channels. Although other heteromeric Kir channels (e.g. Kir3.1/Kir3.4) also exhibit time-dependent activation, the activation of the Kir4.1/Kir5.1 channels is on a much slower time scale and takes several seconds to reach a steady-state current at –120 mV ($\tau = 2$ s

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	Kir4.1-H190		Kir5.1-R230		Kir4.1-E310
	*		*		*
Kir1.1 198	A V I S K R G G K L C L L	232	T I T P E G E T I I	319	V L W G Y R F V P I V
Kir2.1 199	A V I A M R D G K L C L M	233	R I T S E G E Y I P	320	I L W G H R Y E P V L
Kir3.1 200	A V I S M R D G K L T L M	234	R Q T P E G E F L P	321	V L W G H R F F P V I
Kir4.1 185	A V V A Y H N G K L C L M	221	H Q T K E G E N I R	305	I L W G Y E F T P A I
Kir4.2 184	A V I S K Q N G K L C L V	220	H V T K E G E R I L	304	I Y W G F E F V P V V
Kir5.1 188	A L I G M R D G K L C L M	224	S E D S E G R M T M	304	I L W G H R F H D V L
Kir6.2 187	A V I T L R H G R L C F M	221	T T S P E G E V V P	309	I L W G Q R F V P I V

FIG. 1. **Alignment of the C termini of different Kir channels.** Identical residues are shaded *dark gray* and similar residues *light gray*. The alignment reveals that Kir5.1 differs at a key position (Arg-230), which is highly conserved in other Kir channels. Residues that Arg-230 may interact with also differ in Kir4.1 and Kir5.1 and are highlighted with an *asterisk* (*).

compared with 300 ms for Kir3.1/Kir3.4) (15).

The mechanism by which heteromultimerization between Kir4.1 and Kir5.1 produces such novel biophysical properties remains unclear, neither is it understood how Kir5.1 influences the pH sensor in Kir4.1. In this study we have identified an intersubunit interface between the intracellular domains of Kir4.1 and Kir5.1, which influences the inward rectification, time-dependent activation, and pH sensitivity of Kir4.1/Kir5.1 channels. The results also reveal a functional linkage between these different properties and demonstrate how dynamic interactions between the intracellular domains can influence Kir channel permeation, gating, and regulation.

MATERIALS AND METHODS

Molecular Biology—All channel subunits were subcloned into the oocyte expression vector pBF, which provides 5' and 3' untranslated regions from the *Xenopus* β -globin gene flanking a polylinker containing multiple restriction sites. Kir subunits were joined in tandem as previously described (14). This method controls the stoichiometry of the heteromeric channels and does not affect channel function (7, 9, 14). Site-directed mutagenesis was performed using the QuikChange XL protocol (Stratagene). Capped mRNAs were synthesized *in vitro* by using the SP6 mMESSAGE mMACHINE kit (Ambion).

Molecular Modeling—Coordinates of the Kir3.1 structure (Protein Data Bank code 1N9P) were used to produce the models shown in Fig. 5. Visualization and rendering of the structure was done using the RasTop program.¹

Electrophysiology: Two-electrode Voltage-Clamp Recording—*Xenopus laevis* care and handling were in accordance with the highest standards of institutional guidelines in compliance with both national and international laws and policies. Frogs underwent no more than two surgeries, separated by at least 3 weeks. Frogs were anesthetized with an aerated solution of 3-aminobenzoic acid ethyl ester. Standard recording solution contained 90 mM KCl, 3 mM MgCl₂, 10 mM HEPES (pH 7.4) unless otherwise stated. Intracellular acidification was achieved using a potassium acetate buffering system (16). Only one pH/inhibition value per cell was determined in two-electrode voltage-clamp experiments. Measurement of pH sensitivity using this method was less accurate than measurement in excised patches because of the activity of Kir4.1/Kir5.1 channels in the pH 7.5–8.0 range and the inability to alkalize the intracellular pH of the oocytes. Nevertheless, the values recorded provide a quantitative indication of the response to acidification below pH 7.5. Microelectrodes were filled with 3 M KCl and had resistances of 0.1–0.5 megaohms. Recordings were performed at 22 °C, 18–48 h after injection with a GeneClamp 500 amplifier (Axon Instruments) interfaced to a Power Macintosh 7200/90 computer with an ITC-16 computer interface (Instrutech Corp., New York). Currents were evoked by voltage commands from a holding potential of –10 mV, delivered in –10 mV increments from +50 mV to –120 mV, unless otherwise stated. The inward rectification factor (F_{ir}) represents the inward current at –50 mV divided by the outward current at +50 mV ($E_{rev} \sim 0$ mV). Data were given as mean \pm S.E. Results were reproducible in three different batches of oocytes.

RESULTS

A Mutation in Kir5.1 Influences the Time-dependent Activation of Kir4.1/Kir5.1 Channels—To understand the structural

features of Kir5.1 that contribute to the novel properties of heteromeric Kir4.1/Kir5.1 channels we examined an alignment of different Kir channel sequences. This alignment (Fig. 1) reveals that Kir5.1 has a positively charged arginine residue at position 230, whereas all other known Kir channels possess a negatively charged glutamate. We therefore mutated this residue in Kir5.1(R230E) and coexpressed this with Kir4.1 as a Kir4.1-Kir5.1(R230E) tandemly linked dimer. Macroscopic whole cell currents were then recorded by two-electrode voltage-clamp from *Xenopus* oocytes expressing Kir4.1-Kir5.1(R230E). Fig. 2A shows that wild-type Kir4.1/Kir5.1 channels exhibit characteristic time-dependent activation at hyperpolarizing potentials and strong inward rectification. By contrast, the mutant Kir4.1-Kir5.1(R230E) channels exhibit no time-dependent activation and weaker rectification (Fig. 2, B and C). Similar effects were also observed with Kir4.1-Kir5.1(R230D) channels (Fig. 2, D and F). When Arg-230 was substituted with an equivalent positively charged lysine, the Kir4.1-Kir5.1(R230K) channels demonstrated time-dependent activation similar to wild-type but with weaker rectification (Fig. 2, E and F).

Time-dependent Activation and Rectification of Kir4.1/Kir5.1 Channels Is pH Sensitive—Kir4.1-Kir5.1 channels are sensitive to inhibition by intracellular acidification. To monitor the response of the channels to intracellular acidification we utilized a well established potassium acetate buffering system to modulate intracellular pH (16). Whole cell currents were then recorded by two-electrode voltage-clamp from oocytes expressing either wild-type or Kir4.1/Kir5.1(R230E) mutant channels. Fig. 3B shows that there is no difference between the inhibition of the wild-type and mutant Kir4.1-Kir5.1(R230E) channels (pK_a Kir4.1-Kir5.1 = 6.78 ± 0.04 , $n = 6$, pK_a Kir4.1-Kir5.1(R230E) = 6.79 ± 0.04 , $n = 6$).

However, Fig. 3A shows that the slow time-dependent activation of the Kir4.1-Kir5.1(R230E) channels is pH-sensitive and reappears upon intracellular acidification. At pH 7.2 the channels exhibit no time-dependent activation, whereas at pH 6.8 the time-dependent component of activation reappears. The Kir4.1-Kir5.1(R230E) channels also exhibit stronger rectification at pH 6.8 (Fig. 3C). Because overall channel activity decreases with acidification we calculated an inward rectification “factor” (F_{ir}) to compare inward rectification at different pH values (17). F_{ir} represents the inward current at –50 mV divided by the outward current at +50 mV. Thus a larger F_{ir} value represents stronger inward rectification. Fig. 3D shows that for the Kir4.1-Kir5.1(R230E) channels F_{ir} increases by a factor of 3 between pH 7.2 and 6.8 (F_{ir} pH 7.2 = 1.11 ± 0.10 , $n = 6$; F_{ir} pH 6.8 = 3.36 ± 0.50 , $n = 6$).

Fig. 4A shows that the time-dependent activation of both Kir4.1-Kir5.1(R230E) and Kir4.1-Kir5.1(R230D) channels “reappears” in response to intracellular acidification. Furthermore, it shows that the time-dependent activation of the wild-type Kir4.1-Kir5.1 channels is also pH-sensitive. The relative

¹ www.geneinfinity.org/rastop.

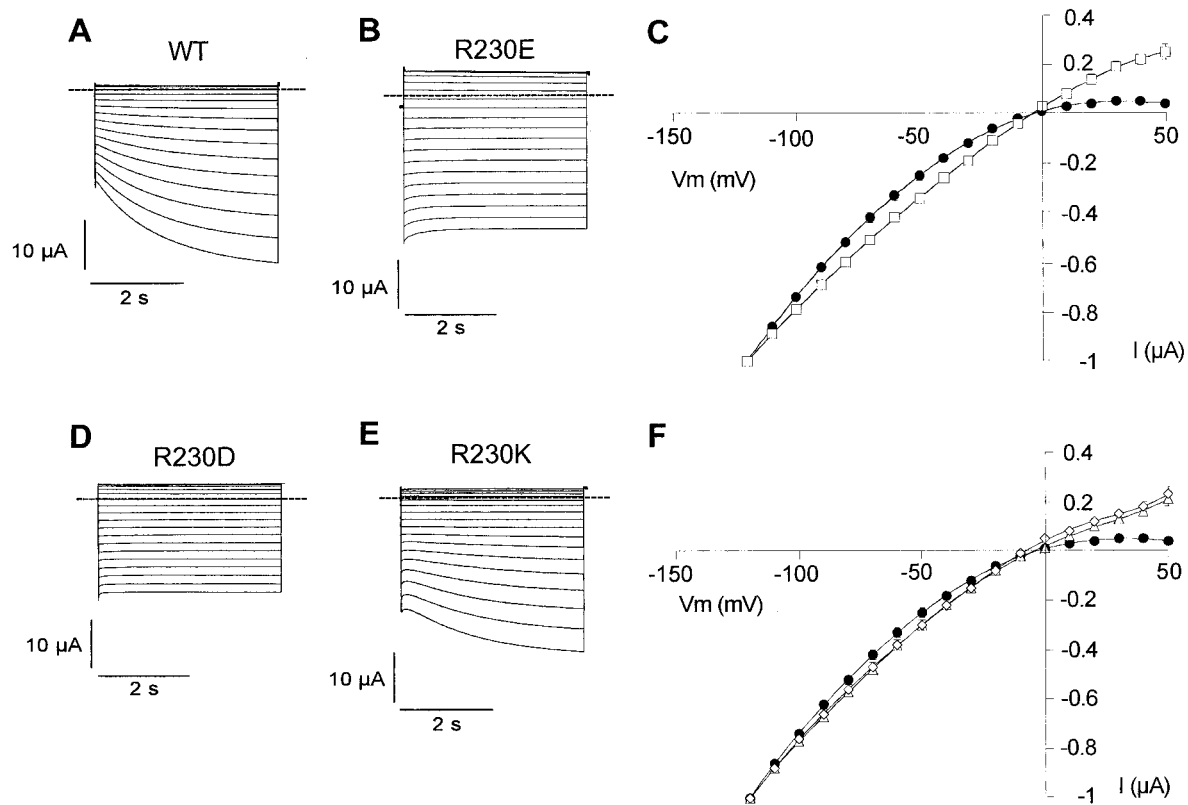


FIG. 2. Mutation of Arg-230 in Kir5.1 alters inward rectification and gating. Representative current families from oocytes expressing Kir4.1/Kir5.1 (A) and Kir4.1-Kir5.1(R230E) (B) mRNAs. The membrane potential of the cell was clamped at -10 mV and the currents were elicited by voltage commands from $+50$ to -120 mV every 10 mV. The dashed line shown in this and all subsequent current traces indicates the zero current level. C, steady-state current-voltage relationship for Kir4.1-Kir5.1 (●) and Kir4.1-Kir5.1(R230E) channels (□). The superimposed current-voltage relationships were normalized to the steady-state current recorded at -120 mV. Representative current families from oocytes expressing Kir4.1-Kir5.1(R230D) (D) and Kir4.1-Kir5.1(R230K) (E) mRNAs. F, steady-state current-voltage relationship for Kir4.1-Kir5.1 (●), Kir4.1-Kir5.1(R230D) (△), and Kir4.1-Kir5.1(R230K) (◇). The data points are the mean \pm S.E. ($n = 6$), which are often smaller than the symbols.

rates of activation for all these channels are shown in Fig. 4B. By contrast there is no effect of pH on the rectification of wild-type Kir4.1-Kir5.1 channels, although the Kir4.1-Kir5.1(R230D) channels demonstrate an increase in rectification at pH 6.8 (Fig. 4C).

Arg-230 in Kir5.1 Forms an Intersubunit Interaction with Kir4.1—While conducting these studies a three-dimensional crystal structure of the intracellular domains of the Kir3.1 channel was resolved (18). The extensive homology between the C termini of the Kir channels means that the Kir3.1 structure can be used to interpret the arrangement of the intracellular domains of all Kir channels. The equivalent residue to Arg-230 in Kir5.1 is Glu-242 in Kir3.1. Fig. 5A shows that Glu-242 in Kir3.1 resides at the end of an extended “arm” on the C terminus. In the tetramer this arm forms close contacts with the adjacent C terminus and this is shown in Fig. 5B. This shows that Glu-242 in Kir3.1 is in close contact with Arg-326 and Arg-205 in the adjacent subunit. In a heteromeric Kir4.1/Kir5.1 channel these residues would reside on the Kir4.1 subunit and are represented by His-190 and Glu-310, respectively. It is interesting to note that His-190 resides within a small domain that we recently identified as a critical determinant of heteromeric assembly between Kir4.1 and Kir5.1 (13).

The alignments in Fig. 1 show that at position 242 in Kir3.1 all known Kir5.1 channels have a glutamate, whereas Kir5.1 has an arginine (Arg-230). Similarly at position 326 in Kir3.1, all known Kir channels have an arginine residue, whereas Kir4.1 and Kir4.2 have a glutamate. Because both Kir4.1 and Kir4.2 exhibit selective heteromultimerization with Kir5.1 it is possible that residues Glu-242 and Arg-326 in Kir3.1 represent an ion pair that is simply reversed in Kir4.1/Kir5.1 channels and

which may contribute to their selective heteromeric assembly. In this case we would expect the Kir4.1(E310R) mutation to have the same functional consequences as the Kir5.1(R230E) mutation. We therefore tested the effect of a charge reversal at this position (E310R). However, the Kir4.1(E310R)/Kir5.1 channels behaved very similar to wild-type with strong rectification and time-dependent activation at pH 7.2. The pH sensitivity of these channels was also identical to wild-type (data not shown). Thus disruption of this putative ion pair by the R230E mutation is unlikely to control the pH-sensitive time-dependent activation of these channels.

Control of pH Sensitivity by an Intersubunit Interaction—Fig. 5B shows that Glu-242 in Kir3.1 also makes close contact with Arg-205. The charged carboxyl group of Glu-242 and the amino group of Arg-205 are less than 4 \AA apart. In other Kir channels the equivalent residue to Kir3.1(R205) is either an arginine or lysine residue, but in Kir4.1 it is a histidine (His-190) and in Kir4.2 a glutamine (Gln-189) (Fig. 1).

We therefore tested the effect of mutating His-190 in Kir4.1. Fig. 6A demonstrates that the Kir4.1(H190R)-Kir5.1 channels exhibit very weak rectification at pH 7.2 and no time-dependent activation. However, as shown in Fig. 6B, at pH 6.8 the rectification increases (Fir pH 7.2 = 1.8 ± 0.20 , $n = 6$; Fir pH 6.8 = 3.6 ± 0.5 , $n = 6$) and the slow time-dependent activation reappears ($\tau = 1.59 \pm 0.20$ s, calculated at -120 mV, $n = 5$). Furthermore, the Kir4.1(H190R)-Kir5.1 channels were significantly less sensitive to inhibition by intracellular acidification (Fig. 7). The pK_a for inhibition of Kir4.1(H190R)-Kir5.1 channels using this method was 6.23 ± 0.01 , $n = 6$.

To examine the potential interaction between Arg-230 in Kir5.1 and His-190 in Kir4.1 we also tested the effect of com-

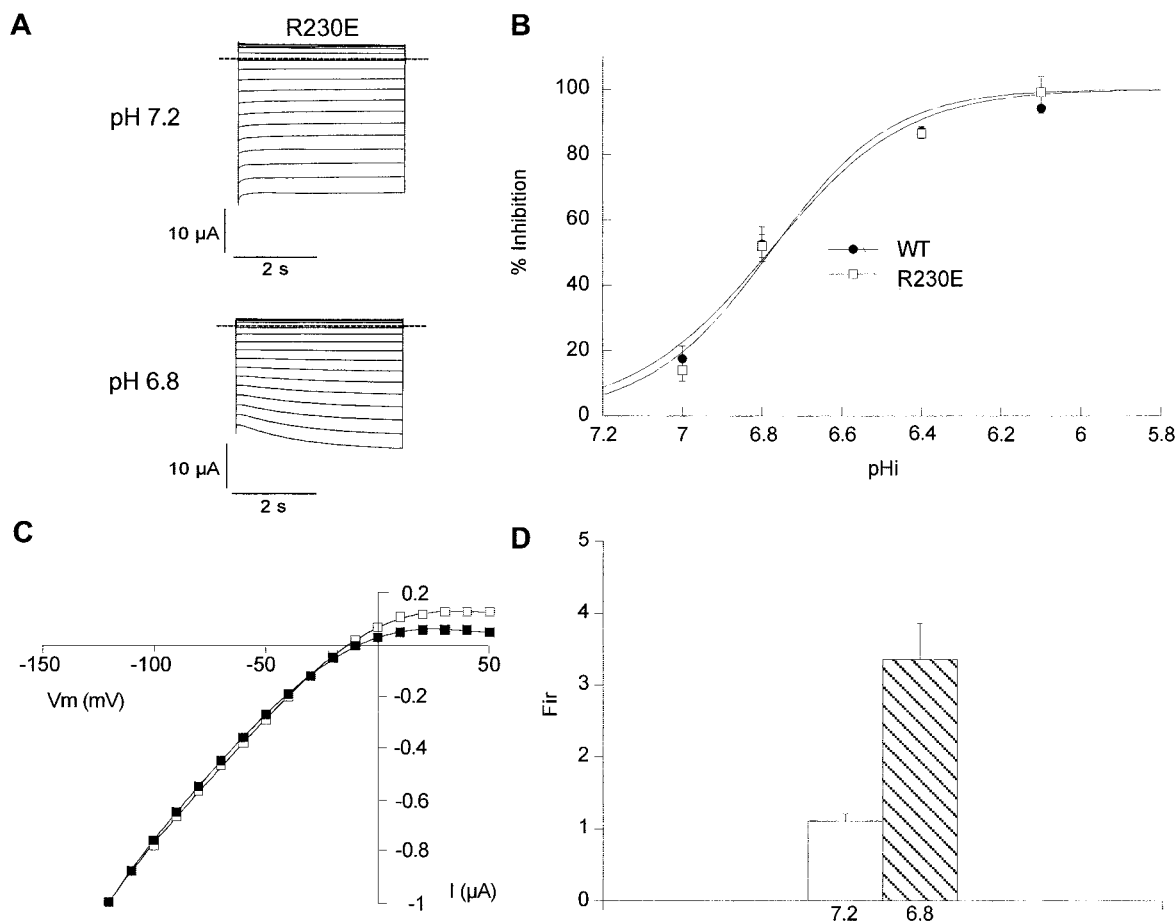


FIG. 3. Inward rectification and gating are pH sensitive. Intracellular acidification modulates the time-dependent activation and the inward rectification of Kir4.1/Kir5.1(R230E) channels. *A*, representative current families of Kir4.1/Kir5.1(R230E) channels recorded at pH 7.2 (upper panel) and 6.8 (lower panel). *B*, presumed intracellular pH versus current inhibition for Kir4.1/Kir5.1 and Kir4.1/Kir5.1(R230E) channels. The currents were recorded at -100 mV in control conditions during the perfusion of a membrane-permeable potassium acetate buffer that reduces the oocyte intracellular pH to the indicated value. Data points are the mean \pm S.E. of 6 oocytes. The solid line shows the fit with the equation: $1/[1 + ([H^+]i/K)n]$ from which the apparent pK_a values were calculated. *C*, normalized current-voltage relationship for Kir4.1-Kir5.1(R230E) recorded at pH 7.2 (\square) and 6.8 (\blacksquare). *D*, inward rectification factor (Fir) for Kir4.1-Kir5.1(R230E) channels calculated at pH 7.2 (open bar) and 6.8 (striped bar); mean \pm S.E., $n = 6$.

binning mutations at both these positions. Fig. 8, *A* and *B*, shows that the Kir4.1(H190R)-Kir5.1(R230E) channels exhibit weak rectification and very little time-dependent activation, and that intracellular acidification induces stronger rectification and time-dependent activation. However, compared with Kir4.1(H190R)-Kir5.1 channels the double mutant exhibits a greater sensitivity to inhibition by H^+ ; pK_a Kir4.1(H190R)-Kir5.1(R230E) = 6.83 ± 0.02 , $n = 5$ (Fig. 8C). Thus the Kir5.1(R230E) mutation has the ability to reverse the loss of pH sensitivity caused by the Kir4.1(H190R) mutation.

DISCUSSION

In this study we demonstrate that residues at an interface between the cytoplasmic domains of Kir4.1 and Kir5.1 influence the permeation, gating, and pH sensitivity of heteromeric Kir4.1-Kir5.1 channels. This intersubunit interaction presents a structural mechanism for the functional coupling of these properties and explains how specific heteromeric interactions can contribute to the novel functional properties observed in heteromeric Kir channels. The highly conserved nature of this structural association throughout the Kir family has implications for understanding the general mechanisms of Kir channel gating and for their regulation by intracellular pH.

A Structural as well as Functional Interaction—The ability of K^+ channels to heteromultimerize contributes to the rich

functional diversity produced by this gene family. However, the interactions that control Kir channel assembly are still poorly understood and no single domain has been shown to regulate channel assembly. Nevertheless, we have recently been able to define a small domain within the proximal C terminus of Kir4.1 that contributes to the highly selective heteromeric interaction between Kir4.1 and Kir5.1 (13). It was therefore of extreme interest to notice that this domain in Kir4.1 contains His-190 and thus contributes to the novel functional properties of Kir4.1/Kir5.1 as well as influencing subunit assembly. However, His-190 in Kir4.1 cannot directly control the heteromeric assembly between Kir4.1 and Kir5.1 because Kir4.2 also shows similar heteromeric specificity and contains a glutamate at this position. Chimeric analysis of this region suggests that other residues within this domain are also required (13). Nevertheless, the overall domain containing His-190 clearly contributes to the specificity of heteromeric assembly and suggests that this interface between Kir4.1 and Kir5.1 serves a dual purpose as both a structural and functional interaction.

It is also important to note that the “extended arm” structure that contains residue Arg-230 in Kir5.1 (see Fig. 5A) is very highly conserved and that a similar interaction must occur in all other Kir channels that are identical to that pictured in Fig. 5B. It is therefore likely that this conserved interface between

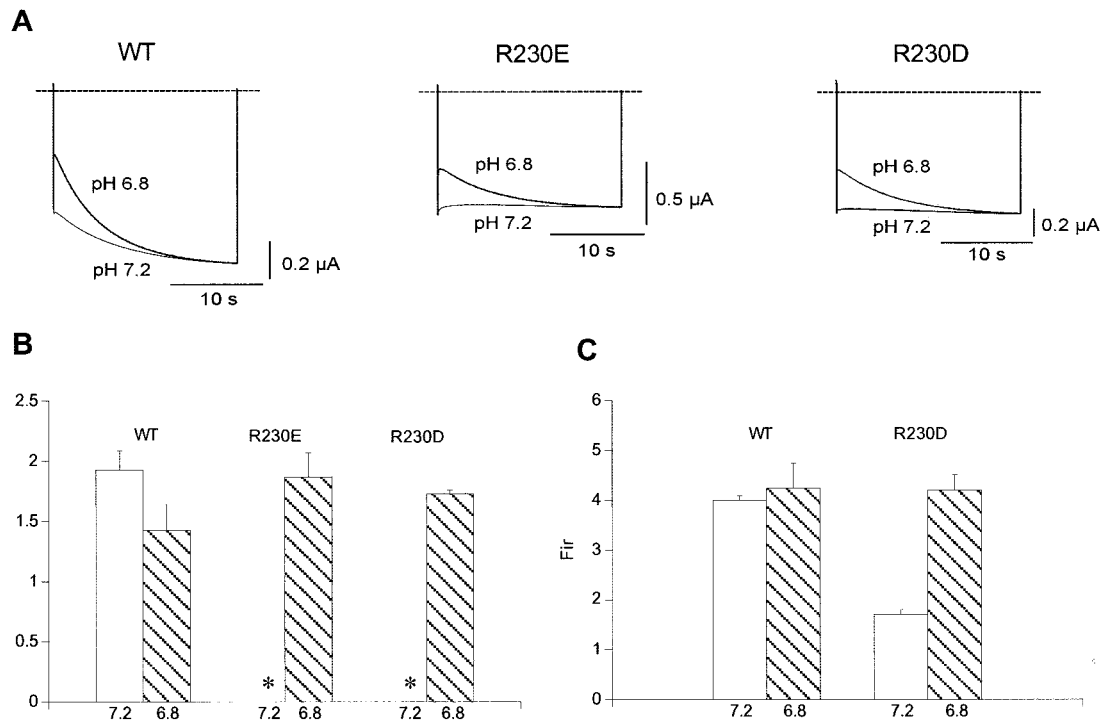


FIG. 4. Time-dependent activation of wild-type Kir4.1-Kir5.1 channels is pH-sensitive. *A*, superimposed and normalized current traces recorded at -120 mV from oocytes expressing Kir4.1/Kir5.1 (left-hand panel), Kir4.1/Kir5.1(R230E) (central panel), and Kir4.1/Kir5.1(R230D) (right-hand panel) at pH 7.2 and 6.8. *B*, the time constant of the slow component was calculated by fitting of the relevant portion of the current trace, recorded at -120 mV, with a single exponential function. The time constants were determined at pH 7.2 (open bar) and 6.8 (striped bar) for the channels reported above each pair of bar graphs (mean \pm S.E., $n = 6$). The asterisk indicates that the time constants were not calculated as these currents show no slow component. *C*, inward rectification factor calculated at pH 7.2 (open bar) and 6.8 (striped bar) for the indicated channels (mean \pm S.E., $n = 6$).

Kir subunits has been adapted by Kir4.1 and Kir5.1 to assist with structural specificity during assembly as well as contributing to its unique functional properties.

Interaction of the Cytoplasmic Domains in Heteromeric Kir Channels—If heteromeric Kir channels adopt a 2 + 2 stoichiometry with subunits opposite each other (14), then for channels where the two subunits are almost identical (e.g. Kir2.1/Kir2.4 (19) and Kir6.1/Kir6.2 (20)) the heteromeric channel is unlikely to be markedly different. Whereas if the two subunits are dissimilar (e.g. Kir4.1/Kir5.1) then the heteromeric channels are more likely to be asymmetric, have novel interactions, and thus novel properties.

The three-dimensional crystal structure of the Kir3.1 intracellular domains (18) and more recently the prokaryotic Kir-Bac1.1 structure (21) demonstrate that these intracellular domains intimately associate with each other to generate an “extended pore” structure. Gating of Kir channels by intracellular ligands such as ATP, G-proteins, and pH have all been shown to involve allosteric movement of these domains (22–26). They are therefore not static but dynamic structures and consequently the contacts between must also be dynamic. We propose that the intersubunit interface we have identified affects channel activity by influencing the interaction between Kir4.1 and Kir5.1 and that such interactions are likely to underlie the regulation of Kir channels by intracellular ligands.

Coupling of Inward Rectification and Time-dependent Activation—There is a functional interdependence between the pore and gating mechanism of many potassium channels. Almost by definition, any gating mechanism will act to obstruct the pore and will therefore interact mechanically with the permeation pathway, also mutations within the pore of several K^+ channels have been shown to influence gating (15, 27, 28). Rectification of Kir channels involves voltage-dependent block by intracellular Mg^{2+} and polyamines (29), and it has been

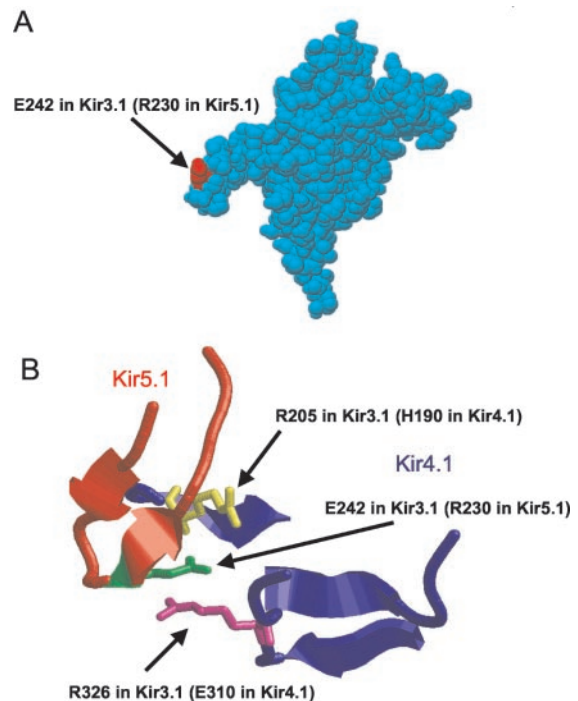


FIG. 5. Kir3.1 structure reveals that Kir5.1(R230) is part of an intersubunit interaction. *A*, individual atoms in the Kir3.1 (GIRK) intracellular domain are rendered as Van der Waal’s spheres and Glu-242 (the residue equivalent to Kir5.1(Arg-230)) is highlighted in red. *B*, Glu-242 is at the end of an extended arm which makes contacts with the adjacent C terminus. Backbones of adjacent C termini are differentially colored red and blue. Key residues are shown and labeled; green, Kir3.1(Glu-242) (Arg-230 in Kir5.1); yellow, Kir3.1(Arg-205) (His-190 in Kir4.1); magenta, Kir3.1(Arg-326) (Glu-310 in Kir4.1).

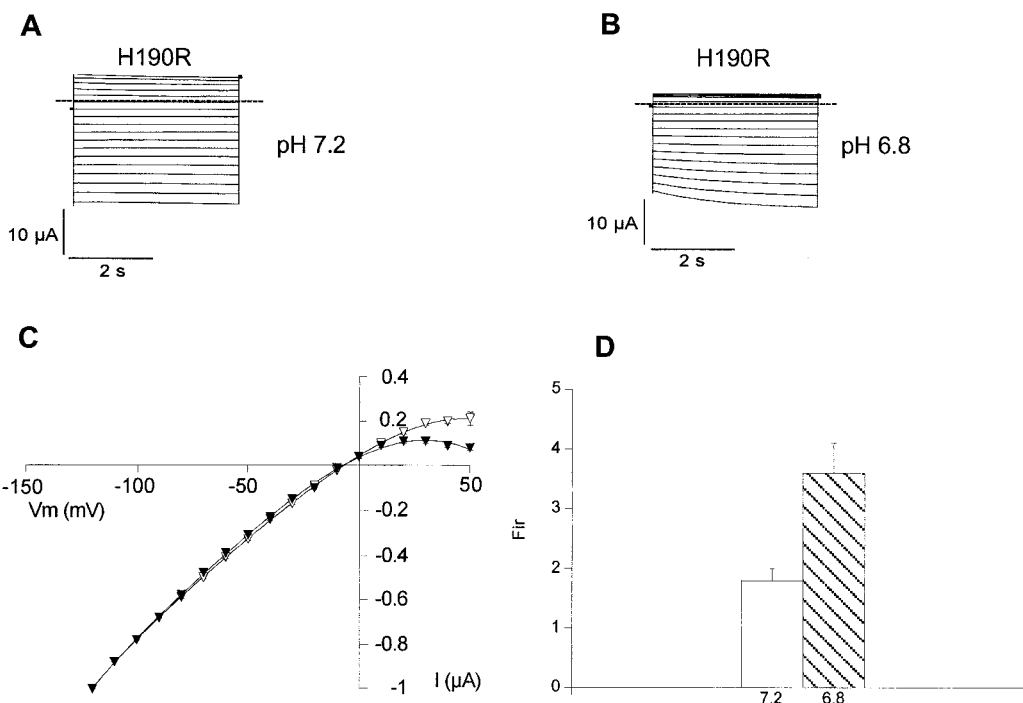


FIG. 6. Mutation of His-190 in Kir4.1 alters inward rectification and gating. Representative current families from oocytes expressing Kir4.1(H190R)-Kir5.1 mRNA recorded at pH 7.2 (A) and 6.8 (B). C, normalized current-voltage relationships for Kir4.1(H190R)-Kir5.1 currents recorded at pH 7.2 (∇) and 6.8 (\blacktriangledown); mean \pm S.E., $n = 6$. D, inward rectification factor for Kir4.1(H190R)-Kir5.1 channels calculated at pH 7.2 (open bar) and 6.8 (striped bar); mean \pm S.E., $n = 6$.

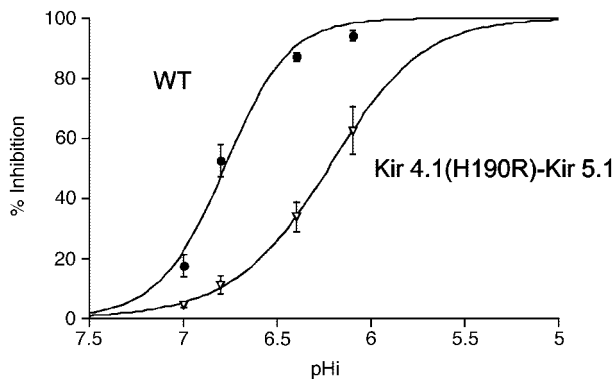


FIG. 7. pH sensitivity of Kir4.1(H190R)/Kir5.1 channels. Plot of the intracellular pH versus current inhibition for oocytes expressing Kir4.1-Kir5.1 (\bullet) and Kir4.1(H190R)-Kir5.1 (∇) mRNAs. Values were calculated as described in the legend to Fig. 3B and under "Materials and Methods." Data points are the mean \pm S.E. $n = 6$.

proposed that the time-dependent component of activation seen in Kir3.0 heteromeric channels is due to the slow unblock of the channel by intracellular polyamines that are bound within this cytoplasmic pore (30). It is therefore possible that polyamines could provide a mechanistic link between the time-dependent activation (or slow unblock) and inward rectification seen in Kir4.1-Kir5.1 channels. However, the Kir4.1-Kir5.1(R230K) mutant exhibits weaker rectification but similar time-dependent activation compared with wild-type channels and if polyamines provide the direct link between these two processes then they would not be so easily uncoupled as they are by this mutation. Also, the R230K mutation demonstrates that it is not simply a reversal of electrostatic charge that disrupts the interaction at this interface. Instead, we propose that the mutations we have identified disturb the dynamic association between adjacent C termini and that this either stabilizes or destabilizes the physical association between the intracellular domains. This will result in allosteric changes in the domains

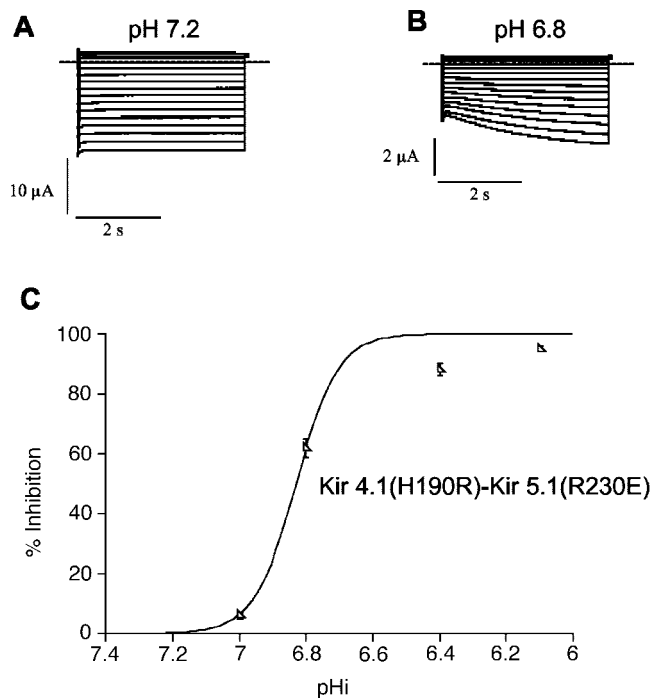


FIG. 8. pH sensitivity of the double mutant Kir4.1(H190R)-Kir5.1(R230E). Representative current families from oocytes expressing Kir4.1(H190R)-Kir5.1(R230E) mRNA recorded at pH 7.2 (A) and 6.8 (B). Note that the time-dependent activation reappears after intracellular acidification. C, pH sensitivity of the Kir4.1(H190R)-Kir5.1(R230E) mutant determined as detailed above. Data points are the mean \pm S.E. of 6 oocytes.

themselves and because they define the inner pore of the channel, and their movement regulates gating, then this provides a mechanism by which intersubunit interactions can control both rectification and gating. The recent KirBac1.1 structure also presents a structural model for how such allosteric rearrange-

ments of the C termini may be translated into channel gating (21). It still remains possible that polyamines may play a role but further experiments will be required to assess their contribution.

pH-sensitive Inward Rectification and Time-dependent Activation—Fig. 3 demonstrates that the inward rectification and time-dependent activation properties of this channel are pH-sensitive. We believe that the pH effect on rectification is most prominent in the Kir4.1-Kir5.1(R230E) mutant because these channels exhibit weaker rectification to start with. By contrast, wild-type channels exhibit strong inward rectification at pH 7.2, therefore it is more difficult to measure any potential increase in rectification induced by acidification. However, pH-dependent effects on the time-dependent activation of wild-type Kir4.1-Kir5.1 channels can still be observed.

But what of the mechanism? Intracellular acidification increases rectification and changes the rate of time-dependent activation, making it more pronounced. The functional effect of the Kir5.1(R230E) mutation is not to abolish this time-dependent activation but to somehow weaken the coupling between this effect and intracellular pH, *i.e.* it appears to shift the pH sensitivity of this process. It is unlikely that direct titration of polyamines accounts for this because their pK_a is generally > 8 . We believe the most likely explanation is that this interaction physically couples the pH-sensing mechanism (primarily on the Kir4.1 subunit) with the stronger rectification and time-dependent activation properties (conferred by the Kir5.1 subunit). Direct titration of histidine 190 in Kir4.1 is also unlikely to be responsible for the pH sensitivity of these properties because the rectification and time-dependent activation of the Kir4.1(H190R) mutant reappear upon intracellular acidification, thus other titratable residues must be responsible. Also, Kir4.2 possesses a glutamine at this position and heteromeric Kir4.2/Kir5.1 channels exhibit similar time-dependent activation and rectification. The interactions at this interface are therefore complex and a simple direct interaction between Kir5.1(Arg-230) and Kir4.1(His-190) cannot explain all these properties. It is therefore more likely that the Kir5.1(R230E) and Kir4.1(H190R) mutations somehow disturb the overall intersubunit interaction between Kir4.1 and Kir5.1 at this interface, and that this weakens the “coupling” between these biophysical properties and changes in intracellular pH.

The Kir4.1(E310R) mutation does not have the same effect as the Kir5.1(R230E) mutation yet in the Kir3.1 structure the equivalent residues seem to be very close. The highly conserved nature of this “ion pair,” which is reversed in Kir4.1/Kir5.1, suggests that it must be of some importance, yet if it were absolutely essential for structural assembly or gating then in the Kir4.1 homomeric channel these residues would both be negatively charged and so would not be able to form an electrostatic ion pair. It was therefore intriguing to note that in a very recent study by Shyng and colleagues (31) this ion pair appears to be responsible for stabilizing the activity of homomeric Kir6.2. However, the equivalent Kir5.1(R230)-Kir4.1(E310) interaction does not appear to contribute to the specific properties we have investigated. Further studies will therefore be required to understand the contribution of this ion pair to heteromeric Kir4.1/Kir5.1 channel function. Nevertheless, the fact that this same interface regulates the activity of Kir6.2 (31) highlights the functional importance of this intersubunit interaction and suggests that similar interactions may contribute to the gating of other Kir channels.

Intersubunit Interactions Influence Inhibition by Intracellular H^+ —One of the most surprising findings was that the Kir4.1(H190R)-Kir5.1 mutation also influences the ability of the channel to sense intracellular H^+ . This observation sug-

gests that the current model for the pH sensor in these channels may be more complex than originally proposed (11, 12). Also, the structure of Kir3.1 and KirBac1.1 reveal that both of the highly conserved arginines thought to produce anomalous titration of the pH sensor lysine are at least 40 Å from the relevant part of TM1, shedding doubt on the precise role of the “RKR triad” in anomalous titration (12). It is possible that H^+ inhibition is influenced by particular gating modes, inhibition occurring preferentially via an open or closed conformation of the channel, and that changes in channel gating produced by particular residues or mutations influence pH sensitivity indirectly. Such indirect effects of mutations on ligand regulation are observed in Kir6.2 where changes in channel gating influence regulation by ATP and H^+ , and also the activation of Kir3.2 by G-proteins (32–34).

It is therefore possible that dynamic interactions between the intracellular domains of Kir4.1 and Kir5.1 influence channel gating and thereby regulation by H^+ . This may also provide a mechanism to account for the fact that physical association with Kir5.1 “enhances” the intrinsic pH sensitivity of Kir4.1, shifting the pK_a into the physiological range. The ability of the Kir4.1(H190R) mutation to affect pH sensitivity, and the ability of the Kir5.1(R230E) mutation to compensate this effect further supports a role for this interface in the control of pH sensitivity. The contribution of this interaction to the pH sensitivity of heteromeric Kir4.2/Kir5.1 channel activity is less significant because Kir4.2 exhibits a much greater pH sensitivity than Kir4.1 (pK_a 7.2 *versus* 6.0) and this is not affected by heteromultimerization with Kir5.1 (10). Nevertheless, Kir4.2 exhibits highly selective heteromultimerization with Kir5.1 and this interface is likely to contribute to this selectivity.

Conclusions—These studies have allowed us to define an interaction between the cytoplasmic domains of Kir4.1 and Kir5.1, which influences inward rectification, gating, and regulation by intracellular pH. Functional coupling of these properties also appears to occur through this interaction. Finally, the fact that this interface is highly conserved and can contribute to the activity of other Kir channels suggests that it may form part of a common gating mechanism.

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