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Molecular determinants for the distinct pH sensitivity of Kir1.1 and Kir4.1 channels

HAOXING XU, ZHENJIANG YANG, NINGREN CUI, LANDE R. GIWA, LATIFAT ABDULKADIR, MONIKA PATEL, PUJA SHARMA, GEORGE SHAN, WANGZHEN SHEN, AND CHUN JIANG
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Xu, Haoxing, Zhenjiang Yang, Ningren Cui, Lande R. Giwa, Latifat Abdulkadir, Monika Patel, Puja Sharma, George Shan, Wangzhen Shen, and Chun Jiang. Molecular determinants for the distinct pH sensitivity of Kir1.1 and Kir4.1 channels. *Am J Physiol Cell Physiol* 279: C1464–C1471, 2000.—Kir1.1 (ROMK1) is inhibited by hypercapnia and intracellular acidosis with midpoint pH for channel inhibition (pK_a) of ~ 6.7 . Another close relative, Kir4.1 (BIR10), is also pH sensitive with much lower pH sensitivity ($pK_a \sim 6.0$), although it shares a high sequence homology with Kir1.1. To find the molecular determinants for the distinct pH sensitivity, we studied the structure-functional relationship using site-directed mutagenesis. An NH_2 -terminal residue (Lys-53) was found to be responsible for the low pH sensitivity in Kir4.1. Mutation of this lysine to valine (K53V), a residue seen at the same position in Kir1.1, markedly increased channel sensitivity to CO_2/pH . Reverse mutation on Kir1.1 (V66K) decreased the CO_2/pH sensitivities. Interestingly, mutation of these residues to glutamate greatly enhanced the pH sensitivity in both channels. Other contributors to the distinct pH sensitivity were histidine residues in the COOH terminus, whose numbers are fewer in Kir4.1 than Kir1.1. Mutation of two of these histidine residues in Kir1.1 (H342Q/H354N) reduced CO_2/pH sensitivities, whereas the creation of two histidines (S328H/G340H) in Kir4.1 increased the CO_2/pH sensitivities. Combined mutations of the lysine and histidine residues in Kir4.1 (K53V/S328H/G340H) gave rise to a channel that had CO_2/pH sensitivities almost identical to those of the wild-type Kir1.1. Thus the residues demonstrated in our current studies are likely the molecular basis for the distinct pH sensitivity between Kir1.1 and Kir4.1.

ion channel; hypercapnia; gene expression; patch clamp; chemoreception

INWARD RECTIFIER POTASSIUM CHANNELS (Kir) play important roles in cellular excitability. Activity of these channels is modulated by various intra- and extracellular substances (11). One of these modulators is a proton that causes inhibition of Kir1 (ROMK), Kir2.3 (HIR), and Kir4.1 (BIR10) (5, 6, 9, 19, 24). The pH sensitivity of Kir1 seems to play a key role in the maintenance of pH and K^+ homeostases by renal epithelial cells (6, 9, 19), while the pH-sensitive Kir4.1 may be a potential molecule for CO_2 chemoreception in

respiratory neurons (21, 22). The Kir1.1 and Kir4.1 share 47% identity and $\sim 75\%$ homology in their amino acid sequences (2, 8). Like other Kir channels, both Kir1.1 and Kir4.1 channels appear to be tetramers with two transmembrane domains (M1 and M2) linked by a pore-forming loop (P or H5 region) containing a signature GYG motif for K^+ channels. Kir1.1 and Kir4.1 also have a Walker-A motif in the COOH terminus, suggesting a potential regulation by ATP. On the basis of many such shared features in Kir1 and Kir4, it has been argued that these two channels should be grouped into one subfamily of Kir channels (12, 15, 18).

Although Kir4.1 and ROMK1 have a number of similarities, intracellular pH (pH_i) sensitivity of Kir1.1 is much higher (midpoint pH for channel inhibition $pK_a \sim 6.7$) than that of Kir4.1 ($pK_a \sim 6.0$). We believe that this difference renders these two channels an optimal pair of subjects for studying structure-functional relationship of Kir channels, since it is possible that the difference is due to small variations in amino acid sequences and their tertiary structures. More importantly, demonstration of molecular determinants for the distinct pH sensitivity may lead to a discovery of novel amino acid residues as well as molecular motifs responsible for channel activity control. Therefore, we designed these experiments in which mutation analyses were carried out on Kir1 and Kir4 using site-directed mutagenesis combined with patch clamp. Our results showed that multiple residues were involved in proton sensing in these Kir channels.

METHODS

Construction of mutants. Kir1.1 (GenBank accession no. X72341) and Kir4.1 (GenBank accession no. X83585) cDNAs were generously provided by Drs. Steven Hebert and John Adelman, respectively (2, 8). These cDNAs were inserted into a eukaryotic expression vector, pcDNA3.1 (Invitrogen, Carlsbad, CA). Site-specific mutations were made using a site-directed mutagenesis kit (Stratagene, La Jolla, CA). Briefly, site-specific mutations were generated using a pair of mutation-containing primers and the *Pfu* DNA polymerase-based polymerase chain reaction. The mutant DNAs were further amplified in the XL1 strain of *Escherichia coli* and purified.

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Correct mutations were confirmed with DNA sequencing. Wild-type (WT) and mutant Kir channels were subsequently expressed in *Xenopus laevis* oocytes.

Preparation and injection of *Xenopus* oocytes. Oocytes were surgically removed from adult frog (*Xenopus laevis*) and treated with 2 mg/ml collagenase (type I, Sigma Chemical, St. Louis, MO) in the OR2 solution containing (in mM) 82 NaCl, 2 KCl, 1 MgCl₂, and 5 HEPES for 90 min at room temperature (~25°C) and pH 7.4. After three washes with the OR2 solution, cDNA in the pcDNA3.1 vector (40–50 ng in 50 nl of double-distilled water) was injected into the oocytes. The oocytes were then incubated at 18°C in the ND-96 solution containing (in mM) 96 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, 5 HEPES, and 2.5 sodium pyruvate plus 100 mg/l geneticin (pH 7.4).

CO₂ exposure and intracellular acidification. *Xenopus* oocytes were placed in a semi-closed recording chamber (Medical System, Greenvale, NY), where perfusion solution bathed both the top and bottom surfaces of the oocytes. The perfusate and the superfusion gas entered the chamber from the inlet at one end and flowed out at the other end. On the top cover of the chamber there was a 3 × 15-mm gap that served as the gas outlet and provided access to the oocytes for recording microelectrodes. The perfusate (KD 90) contained (in mM) 90 KCl, 3 MgCl₂, and 5 HEPES (pH 7.4). At baseline, the chamber was ventilated with atmospheric air. Exposure of the oocytes to CO₂ was carried out by switching the superfusion air to a gas mixture containing 15% CO₂-21% O₂-balance N₂. The high solubility of CO₂ resulted in a detectable change in intra- or extracellular pH as fast as 10 s in these oocytes.

Electrophysiology. Whole cell currents were studied on the oocytes 2–4 days after injection as we described previously (21, 24). In brief, two-electrode voltage clamp was performed using an amplifier (Geneclamp 500; Axon Instruments, Foster City, CA) at room temperature (~25°C). The extracellular solution contained (in mM) 90 KCl, 3 MgCl₂, and 5 HEPES (pH 7.4). Cells were impaled with the use of electrodes filled with 3 M KCl (0.3–0.6 MΩ). Patch-clamp experiments were performed using fire-polished borosilicate glass pipettes. The oocyte vitelline membranes were mechanically removed after exposure to hypertonic solution (400 mosM) for 5 min. The stripped oocytes were placed in FVPP solution [in mM: 40 KCl, 75 potassium gluconate, 5 potassium fluoride, 0.1 sodium vanadate, 10 potassium pyrophosphate, 1 EGTA, 0.2 ADP, 10 PIPES or HEPES, 10 glucose, and 0.1 spermine (pH 7.4)]. Macroscopic current recordings were performed on a giant inside-out patch preparation by using recording pipettes of 0.5–2.0 MΩ (22, 23). The same FVPP solution containing ~150 mM K⁺ was applied to both sides of membranes. In a control experiment, we found that macroscopic currents recorded from giant inside-out patches showed <10% reduction over a 20-min period of recordings in the FVPP solution. Current records were low-pass filtered (2,000 Hz, Bessel, 4-pole filter, -3 dB), digitized (10 kHz, 12-bit resolution), and stored on computer disk for later analyses (pCLAMP 6; Axon Instruments). Nullification of junction potentials between bath and pipette solutions was routinely attempted before seal formation. A parallel perfusion system was used to administer agents to patches at a rate of ~1 ml/min with no dead space (22, 23). Low pH exposures were carried out by using the FVPP solution that had been titrated to various pH levels as required by experimental protocols. HEPES and PIPES buffers were used in these experiments because of their appropriate buffering ranges and membrane impermeability (14).

Data are presented as means ± SE. Differences in means were tested using the Student's *t*-test and ANOVA and were accepted as significant if *P* ≤ 0.05. Channel sensitivity to pH_i in each patch was empirically fitted with the Hill equation in which the apparent pK (pK_a) and the Hill coefficient (*h*) were calculated. To do so, the amplitude of Kir currents was expressed as a function of intracellular pH with the Hill equation $y = 1/[1 + (pK_a/x)^h]$, where *y* is the normalized current amplitude, pK_a is the midpoint pH value for channel inhibition, and *x* is pH_i.

RESULTS

Distinct CO₂ and pH sensitivities exist between Kir1.1 and Kir4.1. Whole cell currents were studied in *Xenopus* oocytes that had received an injection of Kir1.1, Kir4.1, or one of their mutant cDNAs. In the two-electrode voltage-clamp mode, inward rectifying currents as large as 20 μA were observed in most injected oocytes. These currents were sensitive to micromolar concentrations of Ba²⁺ and Cs⁺ (21, 24). Both Kir1.1 and Kir4.1 currents showed evident rectification (Fig. 1, A and B). Exposure of the oocytes to CO₂ (5, 10, and 15%) for 5–8 min produced a reversible and concentration-dependent inhibition of these inwardly rectifying currents (Fig. 1). With 15% CO₂, 70.1 ± 4.0 (*n* = 8) and 23.7 ± 5.6 (*n* = 6) of the whole cell currents were inhibited in WT Kir1.1 and Kir4.1, respectively. These inhibitions did not show evident voltage dependence in a voltage range from -160 to 100 mV (21, 24). The inhibition of Kir1.1 and Kir4.1 currents by CO₂ was mediated by a decrease in pH_i, since selectively lowering pH_i but not extracellular pH (pH_o) to the corresponding pH levels (pH_i 6.6, pH_o 6.2) observed during the 15% CO₂ exposure inhibited both currents to the same degree as hypercapnia did. Macroscopic currents recorded in excised inside-out patches were similarly inhibited (Fig. 2). The pH_i sensitivity of Kir1.1 (pK_a ~6.73, *h* = 3.6), however, is much higher than that of Kir4.1 (pK_a 5.98, *h* = 2.4) (Fig. 2C and Table 1).

Mutation of Lys-67 in Kir4.1 interrupts CO₂/pH sensitivities. Previous studies have shown that Lys-80 in Kir1.1 is critical for pH sensing. Mutation of the Lys-80 into methionine completely eliminates channel sensitivity to pH_i (7). This lysine residue close to the M1 region has therefore been proposed to be a pH sensor in Kir1 channels. In Kir4.1, a lysine (Lys-67) is also found at the same position. We have previously shown that mutation of Lys-67 to methionine, a residue found at the same position in Kir2.1, largely eliminates channel sensitivities to 15% CO₂ and pH_i (21). To further understand this lysine residue in proton sensing, we systematically mutated it to histidine, glutamate, and glutamine as well as methionine (Fig. 3). Although CO₂ sensitivity was lost in K67Q and K67E mutants, the K67H-mutant Kir4.1 was still modestly sensitive to 15% CO₂ (13.1 ± 2.0, *n* = 5). Because this lysine exists in both Kir1.1 and Kir4.1, the difference in their pH sensitivities should depend on other residues.

Lower pH sensitivity in Kir4.1 is related to Lys-53. Thr-51 in Kir1.2 is known to play a role in modulating the pH sensitivity (4). Since this residue is conserved in

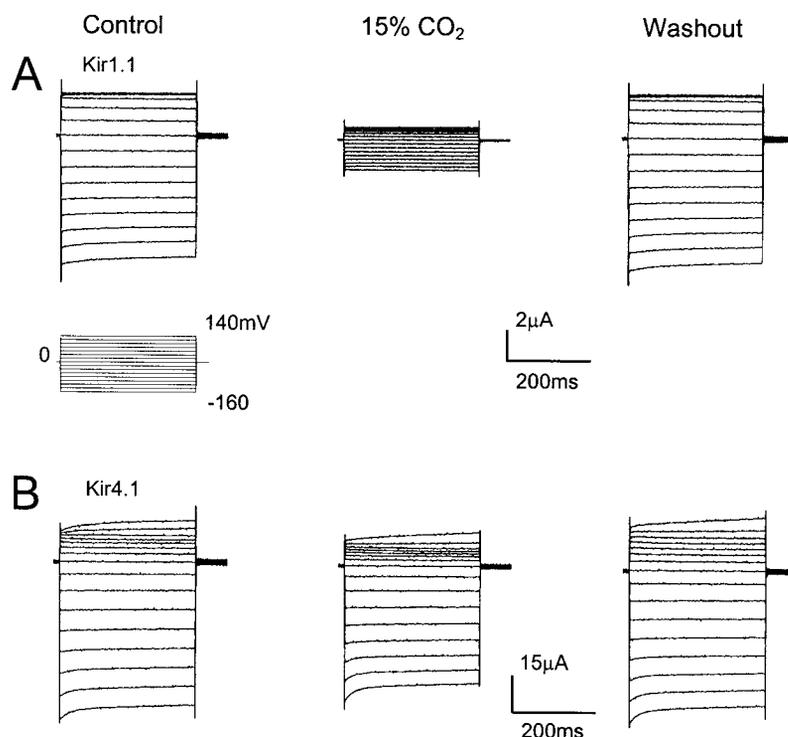


Fig. 1. CO₂ reversibly inhibits Kir1.1 (A) and Kir4.1 (B) currents. Currents were recorded from 2 different oocytes 2 days after cDNA injections. The oocytes were placed 100–200 μm beneath the surface of the bath solution. Currents were recorded in the voltage-clamp mode from –160 to 140 mV with a 20-mV increment using 90 mM K⁺ in the bath solution. The duration of the command pulses was 400 ms for both channels. Current records before, during, and after CO₂ exposure are shown. The duration of CO₂ exposure was 6 and 8 min for Kir1.1 and Kir4.1, respectively. When the inhibition reached a plateau, ~70% Kir1.1 (A) and ~20% Kir4.1 (B) currents were suppressed (measured at –160 mV).

both Kir1.1 (Thr-70) and Kir4.1 (Thr-57), it should not be responsible for the distinct pH sensitivity in these two channels. To determine the role of the NH₂ terminus in pH sensing, we examined the amino acids around this threonine residue. Sequence alignment of positions 64–83 in Kir1.1 and positions 50–69 in Kir4.1 show that two residues clearly contrast their counterparts in the other channel, i.e., Lys-53 and Gln-63 in Kir4.1 vs. Val-66 and Lys-76 in Kir1.1. Mutation of Lys-53 to valine in Kir4.1 (K53V) significantly increased the channel sensitivity to CO₂ (Fig. 3) and pH_i (Fig. 4). The pK_a value of the K53V-mutant Kir4.1 became pH 6.58, which is very close to that of Kir1.1 (pK_a 6.73) (Fig. 5 and Table 1). Subsequently, we reversed the mutation on the Val-66 in Kir1.1. The V66K mutant had a much lower CO₂ sensitivity (20% inhibition by 15% CO₂) than the WT Kir1.1. Recordings from excised patches showed that for this mutant, pK_a = 6.07 and *h* = 3.2 (Figs. 4 and 5). Thus the V66K-mutant Kir1.1 turned more like Kir4.1 than Kir1.1 in terms of its pH/CO₂ sensitivities. In contrast, the Q63K-mutant Kir4.1 showed CO₂ sensitivity (30% inhibition by 15% CO₂) still similar to that of the WT Kir4.1, suggesting that residue Gln-63 does not play a key role in the different pH sensitivity of these channels.

To understand how the valine residue renders a high pH sensitivity to Kir1.1 and how lysine makes the Kir4.1 channels less sensitive to protons, we carried out mutation analyses on Lys-53 in Kir4.1 and on Val-66 in Kir1.1. Mutation of the Lys-53 in Kir4.1 to glutamine (K53Q) had an effect on the CO₂ sensitivity similar to that of the K53V; replacement of the Lys-53 with arginine (K53R) failed to affect the CO₂ sensitiv-

ity in mutant Kir4.1 (Fig. 3). Substitution of the Lys-53 with an acidic residue (K53E) greatly enhanced the CO₂ sensitivity (Fig. 3). Consistent with these observations, recordings from excised patch showed a pK_a of 8.34 in the K53E mutant (Fig. 5). Mutation of Val-66 in Kir1.1 to asparagine (V66N) had no effect on the CO₂ sensitivity (Fig. 3). Substitution of this residue with an acidic amino acid (V66E) shifted the titration curve rightward by 0.43 pH units. Interestingly, both K53E-mutant Kir4.1 and V66E-mutant Kir1.1 had much smaller *h* values than their WT counterparts (Fig. 5 and Table 1). Thus moderate pH/CO₂ sensitivities were observed when a neutral polar or neutral nonpolar residue was present at this position. The pH/CO₂ sensitivities were enhanced with a negative residue and decreased with a positive residue at this position. These results therefore suggest that the status of electrical charge at this site is critical for the channel sensitivity to protons in both Kir1.1 and Kir4.1.

Histidine residues in the COOH terminus are involved in pH sensing in both Kir1.1 and Kir4.1. Although the increase in pK_a value in the K53V-mutant Kir4.1 may have provided some explanations for the lower pH sensitivity of Kir4.1 than Kir1.1, the *h* values of the WT and K53V-mutant Kir4.1 remained significantly low compared with those of the WT and V66K-mutant Kir1.1 (*P* < 0.001). One possibility is that Kir1.1 has more proton-binding sites than Kir4.1 does, an idea supported by our previous studies showing that multiple histidines in the COOH terminus are involved in proton sensing in Kir1.1, mutations of which cause decreases of both pK_a and *h* (3). Thus we studied the histidine residues in the Kir1.1 and Kir4.1 proteins.

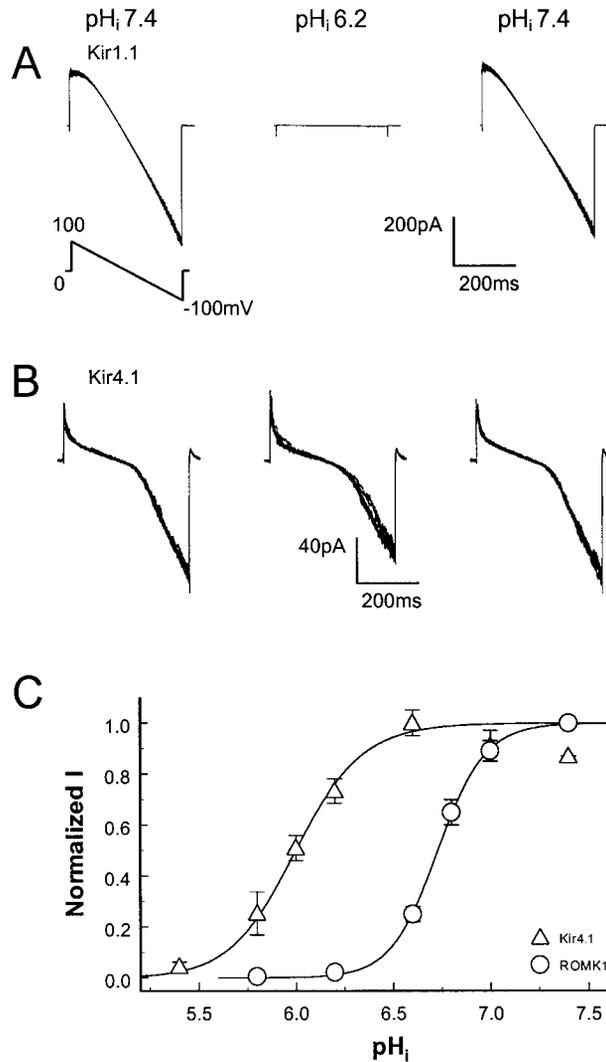


Fig. 2. Inhibitions of Kir currents by protons in excised patches. Kir currents were recorded from inside-out patches with symmetrical K⁺ concentrations (150 mM) applied to both sides of the plasma membranes. Ramp command potentials from 100 to -100 mV were given to the patches at a holding potential of 0 mV. Exposure of the internal membranes to solutions with acidic pH produced a fast and reversible inhibition of inward rectifying currents. Note that 8 superimposed traces are shown in each panel. *A*: Kir1.1 currents were completely inhibited at intracellular pH (pH_i) 6.2. *B*: a slight reduction of Kir4.1 currents was seen at pH_i 6.2. *C*: comparison of concentration-dependent inhibition of Kir4.1 with Kir1.1 currents. The amplitude of these Kir currents is expressed as a function of pH_i using the Hill equation $y = 1/[1 + (pK_a/x)^h]$, where y is the normalized current (I), pK_a is the midpoint pH value for channel inhibition, x is pH_i, and h is the Hill coefficient. The pK_a and h values here are 6.73 and 3.6 for Kir1.1 ($n = 6$) and 6.00 and 2.3 for Kir4.1 ($n = 10$), respectively. Data are presented as means \pm SE.

There are six histidines in the COOH terminus of Kir1.1, but four in that of Kir4.1. To test the hypothesis that Kir1.1 has more proton-binding sites than Kir4.1 in physiological pH range, we created two histidines in Kir4.1 at positions 328 and 340, corresponding to H342 and H354 in Kir1.1. Creation of these histidine residues (S328H/G340H) greatly increased the CO₂ sensitivity of mutant Kir4.1 (Fig. 3). In the S328H/G340H Kir4.1, the titration curve shifted by ~ 0.5 pH units

rightward to a pK_a of 6.50 ($P < 0.001$) with a significant increase in h value ($P < 0.05$) (Figs. 6 and 7; Table 1). When two histidines were mutated to nontitratable residues in Kir1.1 (H342Q/H354N), the pH sensitivity of Kir1.1 changed in exactly the opposite direction ($pK_a = 6.39$ and $h = 3.0$; $P < 0.001$ and $P < 0.05$, respectively). These results are thus consistent with the idea that histidine residues in the COOH terminus of these Kir channels are likely to be proton-binding sites. In an attempt to locate the proton-binding sites in the WT Kir4.1, we studied all histidine residues on the COOH and NH₂ termini of Kir4.1, in which histidines were mutated into nontitratable alanine or glutamine either individually or jointly. While H257Q and H184Q/H190A mutations resulted in nonfunctional channels, all expressed mutants (H221Q, H184Q, and H43A) showed CO₂ sensitivity indistinguishable from that of the WT Kir4.1 (Fig. 3), suggesting that proton-binding sites may be located in either His-190 or His-257 or in other residues titratable at near physiological pH (3, 5, 10).

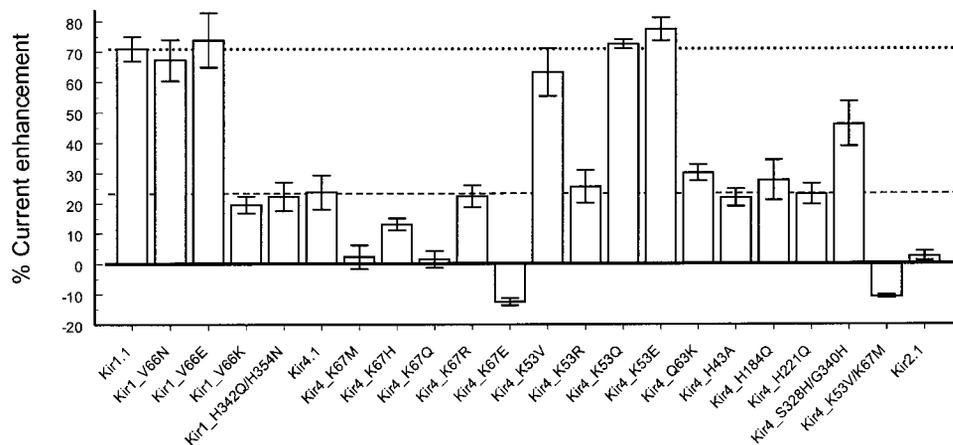
Potential interactions of multiple residues in pH sensing. Our results have indicated that multiple sites are involved in pH sensing in these Kir channels. We noticed that either the K53V mutant or the creation of histidine residues in the COOH terminus alone yielded mutant Kir4.1 that still showed lower pK_a and h values than the WT Kir1.1 did. Thereafter, combined mutations of all these three residues were constructed. The mutant Kir4.1 (K53V/S328H/G340H) had a pH sensitivity very close to that of WT Kir1.1 with a pK_a of 6.78, and the h value (3.0) shifted to a level between that of WT Kir1.1 and WT Kir4.1 (Fig. 7). Combined mutations of some of these residues with Lys-67 in Kir4.1 (K53V/K67M, K67M/S328H/G340H) still showed a low CO₂ sensitivity identical to that of the K67M mutant (Fig. 3). Therefore, the presence of the Val-66 in the NH₂ terminus and additional titratable histidines in the COOH terminus appears to enable higher proton sensitivity in Kir1.1 than in Kir4.1.

Table 1. Proton sensitivity of Kir1.1, Kir4.1, and their mutants

	pK_a	h	n
Kir1.1	6.73 \pm 0.03	3.6 \pm 0.1	6
Kir1_V66E	7.16 \pm 0.03	2.2 \pm 0.2	4
Kir1_V66K	6.07 \pm 0.06	3.2 \pm 0.1	4
Kir1_H342Q/H354N	6.39 \pm 0.03	3.0 \pm 0.2	5
Kir4.1	5.98 \pm 0.03	2.4 \pm 0.2	6
Kir4_K53V	6.58 \pm 0.06	2.3 \pm 0.1	4
Kir4_K53E	8.34 \pm 0.08	1.7 \pm 0.2	4
Kir4_S328H/G340H	6.50 \pm 0.03	3.0 \pm 0.2	4
Kir4_K53V/S328H/G340H	6.78 \pm 0.04	3.0 \pm 0.3	4

Data are means \pm SE; n = no. of patches. Macroscopic currents were recorded using inside-out patches when the intracellular side of membranes was exposed to solutions with various pH levels. These currents were inhibited in a concentration-dependent manner by lowering intracellular pH (pH_i). The inhibitions were described using the Hill equation with apparent pK (pK_a) and Hill coefficient (h) shown. Kir, inward rectifier potassium channels.

Fig. 3. Mutation analyses of CO₂ sensitivity of Kir1.1 and Kir4.1 channel. CO₂ sensitivity was examined using 2-electrode voltage clamp. In Kir1.1, mutations were made on the V66 site and 2 histidines in the COOH terminus. In Kir4.1, mutations were made on K53, K67, S328, and G340, which were replaced by various amino acids of both similar and opposite characteristics. The dotted line indicates the level of CO₂ sensitivity of wild-type (WT) Kir1, and the dashed line indicates that of WT Kir4.1. Data are presented as means ± SE.



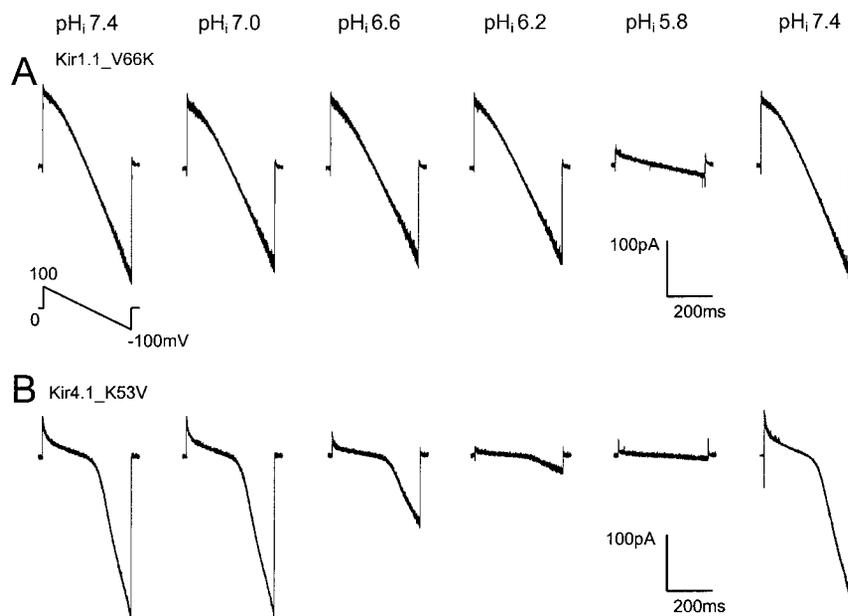
DISCUSSION

Fakler et al. (7) identified a lysine residue close to the M1 in Kir1.1 that is critical for pH sensing in the ROMK1/ROMK2 channel. Mutation of the Lys-80 to methionine completely eliminates the pH sensitivity. Reverse mutation in Kir2.1 introduces the pH sensitivity to the mutant channel (7). At the same position, a lysine residue is also found in Kir4.1. As expected, we found that the K67M- and K67Q-mutant Kir4.1 lost their pH sensitivity. The similar effect of lysine mutation on Kir1.1 and Kir4.1 indicates that this lysine residue is not responsible for the distinct pH sensitivity, though it is involved in certain common processes in channel sensitivity to intracellular protons in both channels.

Previous studies have suggested that a cytoplasmic region in the NH₂ terminus contained a molecular motif modulating the pH sensitivity of ROMK2 (4). This region is known as the "Q" region, which was originally believed to be semi-transmembrane and was later proved to be cytoplasmic. A threonine residue

(Thr-51) in this region is critical for pH sensitivity in ROMK2. Mutation of this residue to alkaline residue lowers pH sensitivity, whereas replacing it with acidic residue enhances channel sensitivity to p*H*_i (4). Since this threonine is conserved in both Kir1.1 and Kir4.1, it should not be the determinant for the distinct pH sensitivity in these channels. Unlike the threonine residue, an adjacent site is not conserved. Indeed, we have found that this site is one of the determinants for the distinct pH sensitivity between these channels. By mutual mutating lysine and valine at this position, we have found that the K53V-mutant Kir4.1 resembles the WT Kir1.1, and the V66K-mutant Kir1.1 appears more like the WT Kir4.1, in terms of pH sensitivity. Hydrophobicity at this site is not critical since the enhanced pH sensitivity remains after mutations to neutral nonpolar (Val) and neutral polar (Gln) residues. Interestingly, a glutamine is found at the same position in Kir4.2 (12). Our results thereby anticipate a higher pH sensitivity in Kir4.2 than in Kir4.1, although the p*K*_a value in the Kir4.2 is still not mea-

Fig. 4. Graded inhibition of the V66K-mutant Kir1.1 and K53V-mutant Kir4.1 by p*H*_i. Macroscopic currents were recorded from inside-out patches under the same conditions as in Fig. 2. *A*: in the V66K-mutant Kir1.1, the current amplitude remained almost identical in a p*H*_i range from 7.4 to 6.2. Further reduction to p*H*_i 5.8 caused almost complete inhibition of these currents. *B*: the K53V-mutant Kir4.1 had much stronger pH sensitivity than WT Kir4.1. The macroscopic currents were suppressed by ~50% at pH 6.6 and by ~90% at pH 6.2.



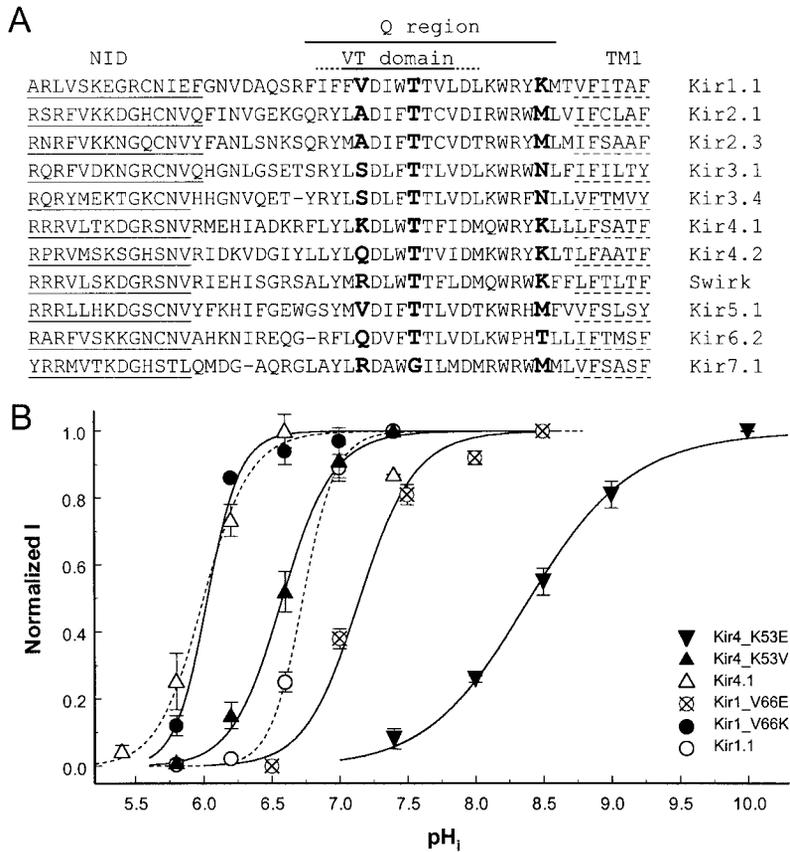


Fig. 5. Identification of critical amino acids in the NH₂ terminus involved in proton sensing. **A:** sequence alignment of 10 Kir channels in the NH₂-terminal region. Residues aligned to Lys-67, Thr-57, and Lys-53 in Kir4.1 are bold. A lysine residue is seen in both Kir1.1 (Lys-80) and Kir4.1 (Lys-67) close to the M1 membrane-spanning sequence (broken underline). In the "Q" region (19), while Thr-57 is conserved in all Kir channels, K53 and Q63 in Kir4.1 are the only two residues showing a clear contrast to their counterparts in Kir1.1. A stretch of amino acids centered by K53 and T57 is designated as VT domain adjacent to the NID domain (solid underline) identified by Tucker et al (20). **B:** pH_i-dependent inhibition of K53 mutants in Kir4.1 and V66 mutants in Kir1.1. The titration curves were constructed as in Fig. 2C. Mutation of Lys-53 to valine (K53V) or glutamate (K53E) shifted the pK_a values rightward by ~0.6 and 2.4 pH units, respectively. Mutations of Val-66 to lysine (V66K) or glutamate (V66E) shifted the pK_a values leftward by 0.6 pH units and rightward by 0.4 pH units, respectively. While the *h* values of K53V- and V66K-mutant Kir4.1 remained the same as those of their WT counterparts, K53E and V66E had significant lower *h* values (see Table 1 for pK_a and *h* values).

sured. Whereas the low pH sensitivity is retained only in the K53R-mutant Kir4.1, substitution of the Lys-53 with an acidic amino acid (Glu) enhances pH sensitivity more strongly than the K53V mutant, suggesting that the status of electrical charge rather than the

species of amino acids at this site plays a role in pH sensing of these Kir channels. This suggestion is further supported by our results from mutations on Val-66 in Kir1.1. High pK_a value in the Kir1.1 is lost in the V66K mutant but maintained in V66N. Similarly to

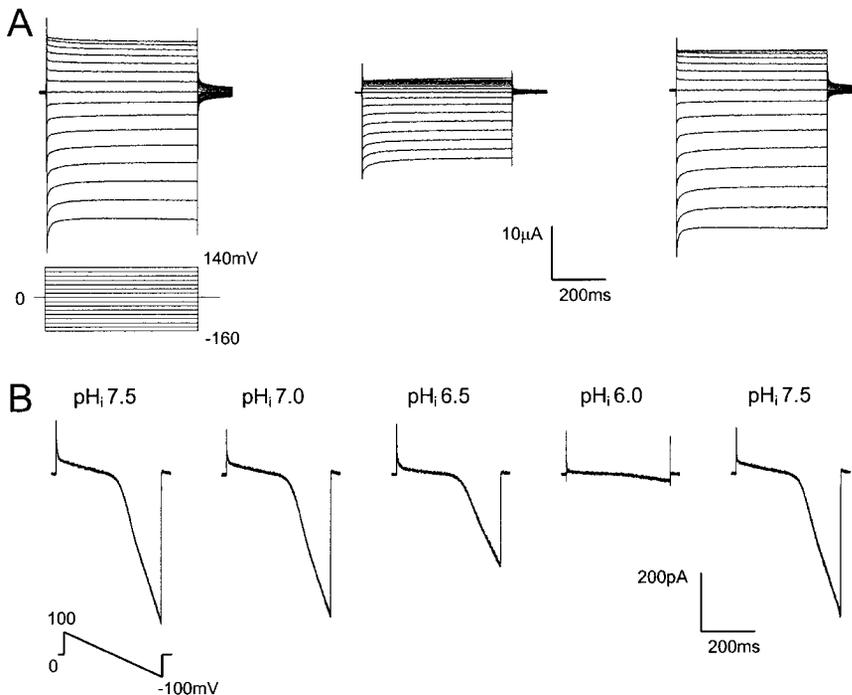


Fig. 6. Increases in CO₂ and pH sensitivities with histidine creations in the COOH terminus of Kir4.1. **A:** whole cell currents were studied in the S328H/G340H-mutant of Kir4.1. Exposure to 15% CO₂ produced an inhibition of these currents by 50%. **B:** in an inside-out patch, the current amplitude started to decrease at pH_i 7.0 and was almost completely suppressed at pH_i 6.0. Washout led to a complete recovery of the currents.

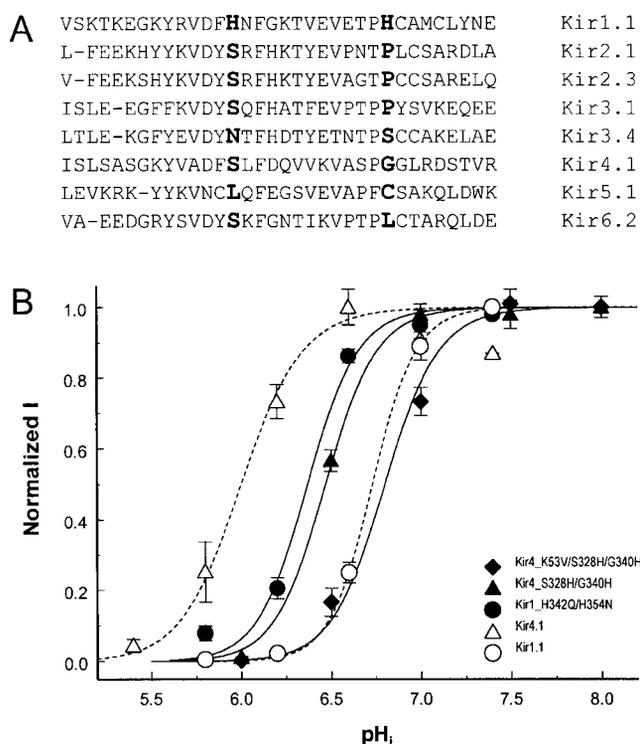


Fig. 7. Involvement of COOH-terminal histidines in proton sensing. **A**: sequence alignment of a region containing potential proton-binding sites in the COOH terminus of 8 Kir channels. In this mildly conserved region, 2 histidine residues are found in Kir1.1 but are absent in Kir4.1 channel. **B**: the effect of histidine mutations on pH sensitivity in Kir1.1 and Kir4.1. Mutations of 2 histidine residues in Kir1.1 (H342Q/H354N) shifted the titration curve leftward by 0.5 pH units (pK_a 6.39, h 3.0) compared with WT Kir1.1 (pK_a 6.73, h 3.6). Creation of 2 histidine residues in Kir4.1 (S328H/G340H), however, shifted the titration curve rightward by 0.5 pH units (pK_a 6.50, h 3.0) compared with WT Kir4.1 (pK_a 5.98, h 2.4). Combined mutations of these histidines and Lys-53 in Kir4.1 (K53V/S328H/G340H) yielded a channel that showed pH_i sensitivity similar to that of Kir1.1.

the K53E-mutant Kir4.1, the V66E mutant shows the highest pH sensitivity.

It is possible that the charged residue interacts with pH sensor(s) and shifts the apparent pK of the sensor or alters the local pH around the sensor by ionic interaction with protons, as suggested by Choe et al. (4). Another potential mechanism that can explain these observations is that positive charges in this region create a repulsive force against other channel domains that may interact with this region and become positively charged at low pH. Apparently, the COOH terminus with several titratable histidine residues is likely to be the counterpart of the interaction. Additionally, both K53E-mutant Kir4.1 and V66E-mutant Kir1.1 show relatively low h values, suggesting that negative charges may increase the attracting force to the COOH-terminal domain so that the NH₂-COOH interaction occurs even before all essential titratable residues in the COOH terminus are protonated.

By introducing potentially titratable histidine residues into Kir4.1 to sites at which potential proton-

binding residues are identified in Kir1.1 (3), we have successfully increased the pK_a level in the S328H/G340H-mutant Kir4.1 and somewhat increased the h value. Moreover, we have found that removal of two histidine residues in Kir1.1 reduces its pH sensitivity, consistent with our previous observations (3). Thus it is likely that the presence of more proton-binding sites in the Kir1.1 contributes to a high pH sensitivity in this channel. Whether there are proton-binding sites other than Lys-67 in Kir4.1 is unclear. On the basis of our data, nonconserved His-221, His-184, and His-43 all contribute little or nothing to the pH sensing. His-190 and His-257 may be the potential candidates. However, mutations of these histidines did not yield functional Kir4.1 channels.

Theoretically, titratable amino acids all can be protonated at certain pH levels if they are fully exposed in the protein tertiary structures. While some of the proton bindings are coupled to the modulation of channel activity, some are linked to other types of channel modulations such as rectification and ligand bindings (1, 9). Hence, multiple proton-binding sites may exist, although the proton bindings may be null given their ability to change channel activity in most cases. Protonation of a single site may cause a great change in channel activity only if this residue is located at a critical area. Otherwise, joint protonations may lead to a significant change in channel activity, as we have shown previously for histidine residues in the COOH terminus of Kir1.1 (3).

Protonation of Lys-80 residue in Kir1.1 has been suggested (7, 16). Since the pK_a value of lysine is 10.78, titration of such a residue requires its interactions with other protein domains (7, 16). Studies have recently shown that the intercellular NH₂ and COOH termini may interact under certain circumstances: both NH₂ and COOH termini are required for gating of Kir channels by protons (14, 16, 17); the intracellular NH₂- and COOH-terminal domains can bind each other in purified NH₂ and COOH peptides (13, 20). It is possible that protonation of histidine residues in the COOH terminus conveys the COOH terminus to a conformation that can interact with the NH₂-terminal domain near to Val-66 and Thr-70 in Kir1.1 (or the VT domain). The NH₂-COOH interaction subsequently causes a change in the pK_a value of Lys-80 or a conformational change of the M1 sequence through the Lys-80 residue, leading to a closure of the Kir channel. Since there are fewer titratable residues in the COOH terminus and a positive charge in the NH₂-terminal domain of Kir4.1, its NH₂-COOH domains may interact poorly, and thus this channel is less sensitive to intracellular acidosis than Kir1.1.

In conclusion, we have demonstrated several molecular determinants for the distinct pH sensitivity in Kir1.1 and Kir4.1. The presence of a lysine (Lys-53) and the lack of two histidine residues in Kir4.1 appear to render the channel lower in pH sensitivity than Kir1.1.

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