Biophysical and Molecular Mechanisms Underlying the Modulation of Heteromeric Kir4.1–Kir5.1 Channels by CO₂ and pH

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Abstract: CO₂ chemoreception may be related to modulation of inward rectifier K⁺ channels (Kir channels) in brainstem neurons. Kir4.1 is expressed predominantly in the brainstem and inhibited during hypercapnia. Although the homomeric Kir4.1 only responds to severe intracellular acidification, coexpression of Kir4.1 with Kir5.1 greatly enhances channel sensitivities to CO₂ and pH. To understand the biophysical and molecular mechanisms underlying the modulation of these currents by CO₂ and pH, heteromeric Kir4.1–Kir5.1 were studied in inside-out patches. These Kir4.1–Kir5.1 currents showed a single channel conductance of 59 pS with open-state probability (P_open) ~ 0.4 at pH 7.4. Channel activity reached the maximum at pH 8.5 and was completely suppressed at pH 6.5 with pKa 7.45. The effect of low pH on these currents was due to selective suppression of P_open without evident effects on single channel conductance, leading to a decrease in the channel mean open time and an increase in the mean closed time. At pH 8.5, single-channel currents showed two sublevels of conductance at ~1/4 and 3/4 of the maximal openings. None of them was affected by lowering pH. The Kir4.1–Kir5.1 currents were modulated by phosphatidylinositol-4,5-bisphosphate (PIP₂) that enhanced baseline P_open and reduced channel sensitivity to intracellular protons. In the presence of 10 μM PIP₂, the Kir4.1–Kir5.1 showed a pKa value of 7.22. The effect of PIP₂, however, was not seen in homomeric Kir4.1 currents. The CO₂/pH sensitivities were related to a lysine residue in the NH₂ terminus of Kir4.1. Mutation of this residue (K67M, K67Q) completely eliminated the CO₂ sensitivity of both homomeric Kir4.1 and heteromeric Kir4.1–Kir5.1. In excised patches, interestingly, the Kir4.1–Kir5.1 carrying K67M mutation remained sensitive to low pH, but pH sensitivity, however, disappeared in the presence of PIP₂. The effect of PIP₂ on shifting the titration curve of wild-type and mutant channels was totally abolished when Arg178 in Kir5.1 was mutated. Thus, these studies demonstrate a heteromeric Kir channel that can be modulated by both acidic and alkaline pH, show the modulation of pH sensitivity of Kir channels by PIP₂, and provide information of the biophysical and molecular mechanisms underlying the Kir modulation by intracellular protons.

Keywords: CO₂ chemoreception • pH • phosphatidylinositol-4,5-bisphosphate • excised patch • brainstem

Introduction

Inward rectifier K⁺ channels (Kir channels) are the primary regulators of membrane excitability and are themselves also regulated by several intracellular factors (Nichols and Lopatin, 1997). One of these factors is a hydrogen ion that has been known to affect specific members in the Kir channel family (Coulter et al., 1995; Tsai et al., 1995; Doi et al., 1996; Shuck et al., 1997; Pearson et al., 1999; Zhu et al., 1999; Xu et al., 2000). The modulation of Kir channels by protons is significant because changes in intracellular pH are seen in a number of physiological and pathophysiological conditions, and because such a modulation may allow cells to produce appropriate responses to these conditions. Indeed, Kir channels with pH sensitivity have been shown to be involved in the maintenance of pH and K⁺ homeostasis by renal epithelial cells (Schlatter et al., 1994; Zhou and Wingo, 1994; Tsai et al., 1995; Doi et al., 1996) and CO₂ chemosensitivity of brainstem neurons (Pineda and Aghajanian, 1997). In brainstem neurons, the inhibition of inward rectifying K⁺ channels causes depolarization and an increase in membrane excitability, which may constitute an initial event in CO₂ chemoreception (Mitchell and Berger, 1975). Thus, detailed studies of these Kir channels may give rise to an understanding of a group of CO₂-sensitive molecules that are located on plasma membranes of nerve cells, detect neuronal ambient PCO₂ levels, and couple the PCO₂ fluctuation to a corresponding change in membrane excitability.

Several cloned Kir channels respond to CO₂ and pH similarly to the K⁺ currents seen in brainstem neurons. Kir1.1 and Kir1.2 are inhibited by a decrease in intracellular pH (Tsai et al., 1995; Doi et al., 1996; Fakler et al., 1996b; Choe et al., 1997; McNicholas et al., 1998)
and hypercapnic acidosis (Chanchevalap et al., 2000; Zhu et al., 2000). A lysine residue near to the first membrane-spanning domain or TM1 (Lys80 in Kir1.1 and Lys61 in Kir1.2) is critical for the pH sensitivity (Fakler et al., 1996b; McNicholas et al., 1998). Hypercapnia and acidosis also inhibit Kir2.3 (Coulter et al., 1995; Zhu et al., 1999, 2000). A threonine residue also located at the immediate vicinity of the TM1 domain (Thr53) plays an important role in channel sensitivity to intracellular protons (Qu et al., 1999). Although these Kir channels are sensitive to CO₂ and pH, they may not be the optimum candidates of the CO₂-sensing molecules for the simple reason that none of them has a high level of expression in the brainstem. Thus, it is uncertain whether these Kir channels are the CO₂/pH sensors in the brainstem neurons.

In contrast, Kir4.1 is expressed predominantly in the brainstem (Bredt et al., 1995). Although its pH sensitivity remains low (apparent pKᵣ or pKa ~ 6.0), coexpression of Kir4.1 with the brain Kir5.1 greatly enhances CO₂ and pH sensitivities of the heteromeric Kir4.1-Kir5.1 channels (Xu et al., 2000). Interestingly, the Kir5.1 that is also expressed in the brainstem (our unpublished observations) does not form functional channels in its own homomultimers. Its only known function is to produce heteromeric channels, particularly with Kir4.1 (Pessia et al., 1996). The high CO₂/pH sensitivities and brainstem-specific expression indicate that these channels are preferential candidates as CO₂-sensing molecules in brainstem neurons. To understand their potential role in CO₂ chemoreception, it is necessary to understand the biological and molecular mechanisms underlying the modulation of Kir4.1-Kir5.1 channels by CO₂ and pH.

Several mechanisms can underlie the modulation of the heteromeric Kir4.1-Kir5.1 by CO₂. Our previous studies have shown that Kir channel inhibition during hypercapnia is mediated by protons rather than molecular CO₂ (Xu et al., 2000; Zhu et al., 2000). The proton-sensing, however, can be an inherent property of the channel proteins, or it can be carried out by another intermediate molecule; the modulation of Kir4.1-Kir5.1 currents can be a result of a depression of single-channel conductance or a reduction in channel open-state probability (P_open); and the proton-sensing mechanisms can be on the Kir4.1 or Kir5.1 subunit, since the lysine and threonine residues found in Kir1.1 and Kir2.3 are also seen in Kir4.1 (Lys67) and Kir5.1 (Thr68), respectively. To examine these possibilities, we performed experiments in which cell-free excised patch and molecular genetic techniques were used to answer these questions.

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Oocytes from female frogs (Xenopus laevis) were used in the present studies. Frogs were anesthetized by bathing them in 0.3% 3-aminobenzoic acid ethyl ester. A few lobes of ovaries were removed after a small abdominal incision (~5 mm). The surgical incision was closed and the frogs were allowed to recover from the anesthesia. Xenopus oocytes were treated with 2 mg/ml of collagenase (Type I; Sigma-Aldrich) in OR2 solution (mM): 82 NaCl, 2 KCl, 1 MgCl₂, and 5 HEPES, pH 7.4, for 90 min at room temperature. After three washes (10-min each) of the oocytes with OR2 solution, cDNAs (25–50 ng in 50 nl double-distilled water) were injected into the oocytes. The oocytes were then incubated at 18°C in ND-96 solution containing (mM): 96 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, 5 HEPES, and 2.5 sodium pyruvate with 100 mg/liter gentamicin added (pH 7.4).

Brain Kir4.1 (BIRK10) and brain Kir5.1 (BIRK9) cDNAs were generously provided by Dr. John Adelman (Organ Health Science University) (Bond et al., 1994). A vector for eukaryotic expression (pcDNA3; Invitrogen) was used to express Kir4.1 and coexpress Kir4.1 and Kir5.1 channels in Xenopus oocytes. The Kir4.1 cDNA was removed from the pBf vector at EcoRI restriction sites on each end of the cDNA, and then were subsequently subcloned into corresponding EcoRI sites in the pcDNA3.1. For coexpression of Kir4.1 and Kir5.1, a tandem dimer of these two cDNAs was constructed using the overlapping extension technique (Qu et al., 1999), in which the full-length Kir4.1 and Kir5.1 sequences were obtained using Pfu DNA polymerase (Stratagene) chain reaction. The PCR products were joined to each other at the 3’ end of Kir4.1 and 5’ end of Kir5.1, and then cloned in the pcDNA3.1, as detailed by Pessia et al. (1996). Site-specific mutations were produced using a site-directed mutagenesis kit (Stratagene) using the tandem dimer of Kir4.1-Kir5.1 as a template. The orientation and site-specific mutations were confirmed with DNA sequencing. Expression of these channels was examined with cDNA injections.

Whole-cell currents were studied on the oocytes 2–4 d after injection. Two-electrode voltage clamp was performed using an amplifier (Geneclamp 500; Axon Instruments Inc.) at room temperature (23–26°C). The extracellular solution contained (mM): 90 KCl, 3 MgCl₂, and 5 HEPES, pH 7.4. Cells were impaled using electrodes filled with 3 M KCl. The potential leakage of KCl from the recording electrodes was not corrected because of the large volume of oocytes. One of the electrodes (1.0–2.0 MΩ) served as voltage recording, which was connected to an HS-1 × 1L headstage (input resistance = 10¹¹ Ω), and the other electrode (0.3–0.6 MΩ) was used for current recording connected to an HS-2 × 10MG headstage (maximum current = 130 μA). Oocytes were accepted for further experiments only if their leak currents, measured as a difference before and after leak subtraction, were <10% of the peak currents. The leak subtraction was applied to oocytes if their leak currents were 5–10%, which was done by subtracting a sum of currents produced by five small depolarizing pulses in 1/5 of command potentials. Current records were low-pass filtered (Bessel, four-pole filter, 3 dB at 5 kHz), digitized at 5 kHz (12-bit resolution), and stored on computer disk for later analysis (PCLAMP 6; Axon Instruments, Inc.) (Yang and Jiang, 1999; Zhu et al., 1999). Junction potentials between bath and pipette solutions were appropriately nullled.

Patch-clamp experiments were performed at room temperature (~25°C) as described previously (Yang and Jiang, 1999; Zhu et al., 1999). In brief, fire-polished patch pipettes (2–4 MΩ) were made from 1.2-mm borosilicate capillary glass (Sutter Instrument Co.). Single channel currents were recorded from inside-out, outside-out, and cell-attached patches (Hamill et al., 1981). Giant inside-out patches were also employed to study macroscopic currents in a cell-free condition using recording pipettes of 0.5–1.0 MΩ. Current records were low-pass filtered (2,000 Hz, Bessel, four-pole filter, ~3 dB), digitized (10 kHz, 12-bit resolution), and stored on computer disk for later analysis (PCLAMP 6; Axon Instruments, Inc.). Junction potentials between bath and pipette solutions were appropriately nullled before seal formation.
For single channel recordings, the oocyte vitelline membranes were mechanically removed after being exposed to hypertonic solution (400 mOsm) for 15 min. The stripped oocytes were placed in a petri dish containing regular bath solution (see below). Recordings were performed using solutions containing equal concentrations of KCl applied to the bath and recording pipettes. The bath solution contained (mM): 40 KCl, 75 potassium gluconate, 5 potassium fluoride, 0.1 sodium vanadate, and 10 potassium pyrophosphate, 1 EGTA, 0.2 ADP, 10 PIPES, 10 glucose, and 0.1 spermine (FVPP solution, pH 7.4). The pipette was filled with the same FVPP solution used in the bath or a solution containing (mM): 40 KCl, 110 potassium gluconate, 0.2 ADP, 1 EGTA, 10 HEPES, 10 glucose, 2 MgCl2, pH 7.4. This bath solution was chosen after several others had been tested regarding channel rundown in excised patches. In a control experiment, we found that macroscopic currents recorded from giant inside-out patches were very well maintained, showing <10% reduction over a 17-min period of recordings in such a bath solution. This period is sufficient for all our single-channel recording protocols that were designed to be completed generally within 10 min.

CO2 exposures were performed in a semi-closed recording chamber (BSC-HT, Medical System), in which oocytes were placed on a supporting nylon mesh, and the perfusion solution bathed both the top and bottom surface of the oocytes. The perfusate and the superfusion gas entered the chamber from two inlets at one end and flowed out at the other end. There was a 3 × 15-mm gap on the top cover of the chamber, which served as the gas outlet and the access to the oocytes for recording microelectrodes. The perfusate contained (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 a perfusate that had been bubbled for at least 30 min with a gas mixture containing CO2 (5, 10, or 15%) balanced with 21% O2 and N2, and superfused with the same gas. The high solubility of CO2 resulted in a detectable change in intra- or extracellular acidification as fast as 10 s in these oocytes. Thus, in most experiments, only the superfusion air was switched to CO2, in which similar results were produced.

A parallel perfusion system was used to administer agents to patches or cells at a rate of ~1 ml/min with no dead space (Zhu et al., 1999). Low pH exposures were carried out using the same bath solutions that had been titrated to various pH levels, as required by experimental protocols. PIPES buffer was used because of its appropriate buffering range and its impermeability to plasma membranes. Indeed, we have done a control experiment in which a recombinant Kir2.3 with Kir2.1 sequence in its NH2-terminal and Kir2.3 sequence in the rest part of the channel protein was expressed in the oocytes (Qu et al., 1999). Currents studied in inside-out giant patches did not show any detectable change to intracellular acidifications, although whole-cell currents of this Kir2.3 mutant were inhibited by extracellular acidification (Qu et al., 1999).

For single-channel analysis, data were further filtered (0–1,000 Hz) with a Gaussian filter. This filtering causes events shorter than 100 μs to be ignored. No correction was attempted for the missed events. Single channel conductance was measured as a slope conductance with at least two voltage points. \( P_{\text{open}} \) was calculated by first measuring the time, \( t_j \), spent at current levels corresponding to \( j = 0,1,2,\ldots \) channels open (Yang and Jiang, 1999; Zhu et al., 1999). The \( P_{\text{open}} \) was then obtained as \( P_{\text{open}} = \left( \sum_j t_j \right) / n \) where \( n \) is the number of channels active in the patch and \( t_j \) is the duration of recordings. \( P_{\text{open}} \) values were calculated from stretches of data having a total duration of 20–200 s. Open and closed times were measured from records in which only a single channel was active. The open- and closed-time distributions were fitted using the method of maximum likelihood (Yang and Jiang, 1999; Zhu et al., 1999). The current amplitude was described using Gaussian distributions and the difference between two adjacent fitted peaks was taken as unitary current amplitude.

Data are presented as means ± SEM (n = number of patches). Differences in means were tested with the Student’s test and were accepted as significant if \( P \leq 0.05 \).

**RESULTS**

**Inhibition of Kir Currents by CO2**

Effects of CO2 on K+ currents were studied using a high K+ (90 mM) bath solution. Membrane potential was held at 0 mV and stepped from −160 to 100 mV in 20-mV increments. Under such a condition, clear inward rectifying currents were observed in the oocytes receiving an injection of Kir4.1 or a tandem-linked Kir4.1 and Kir5.1 (Kir4.1–Kir5.1) cDNA 2–3 d earlier. Exposure of these oocytes to 15% CO2 for 4–6 min produced an inhibition of Kir4.1 and Kir4.1–Kir5.1 currents. The degree of the inhibition was different between these two currents. At the maximal inhibition (measured at −120 mV), the Kir4.1–Kir5.1 currents were suppressed by 59.4 ± 3.7% (n = 9) and the Kir4.1 by only 23.3 ± 4.8% (n = 8). The effect of CO2 on these currents was reversible and depended on CO2 concentrations. Evidence inhibition of these Kir currents was seen with 5% CO2. Higher concentrations of CO2 (10–15%) induced a much stronger inhibition of these currents. This inhibition was likely to be mediated by intracellular acidification, since lowering intra- but not extracellular pH to levels measured during 15% CO2 produced a similar inhibition to 15% CO2. These results are therefore consistent with our previous observations (Xu et al., 2000).

**Baseline Single Channel Properties of Kir4.1–Kir5.1**

To understand channel biophysical properties underlying the effect of protons, single-channel activity was studied in inside-out patches, after the expression of Kir currents was identified in the two-electrode voltage-clamp mode. These patches were exposed to symmetric concentrations of K+ (150 mM) on both sides of plasma membranes; command potentials from −120 to 100 mV were applied to these patches. When inward-rectifying currents were seen, the slope conductance was first measured. Fig. 1 A shows a single-channel current recorded from a Kir4.1–Kir5.1-injected oocyte. The current showed a clear inward rectification with the slope conductance of 59 pS in the inside-out patch configuration (Fig. 1 B). In comparison with the homomeric Kir4.1, the single channel conductance of the Kir4.1–Kir5.1 was much larger, which averaged 59.2 ± 6 pS (n = 17) vs. 22.2 ± 0.5 pS (n = 22) in the Kir4.1 currents. Although the Kir4.1 showed a high baseline channel activity (\( P_{\text{open}} = 0.890 ± 0.021, n = 5 \)), the heteromeric Kir4.1–Kir5.1 channels had a mean \( P_{\text{open}} \) of only 0.416 ± 0.095 (n = 6) at pH 7.4. When the pH
level in the internal solution increased to pH 8.5, a large increase in channel activity was seen (Fig. 2 A), suggesting that channel activity was partially inhibited at physiological pH level. The $P_{\text{open}}$ was not affected by a change in membrane potential from $-100$ to $-40$ mV in both Kir4.1 and Kir4.1–Kir5.1. At pH 8.0 and 8.5, single channel Kir4.1–Kir5.1 currents showed two sublevels of conductance, which were $1/4$ and $3/4$ of the conductance of the full open state (Fig. 2, A and B). No bursting activity was observed at pH 8.0 and 8.5.

At pH 8.5, single-channel Kir4.1–Kir5.1 currents showed long periods of openings and short periods of closures (Fig. 2). The mean open time of these currents was $49.4 \pm 3.6$ ms ($n = 4$), and the mean closed time was $12.6 \pm 4.5$ ms ($n = 4$). Fig. 3, A and B, illustrates dwell-time histograms of the Kir4.1–Kir5.1 currents, in which two components of time constant are revealed for the channel open state and three for the closed state ($\tau_{O1} = 0.9 \pm 0.2$ ms; $\tau_{O2} = 57.0 \pm 3.8$ ms; $\tau_{C1} = 0.3 \pm 0.1$ ms; $\tau_{C2} = 4.0 \pm 0.3$ ms; $\tau_{C3} = 135.5 \pm 53.0$ ms; $n = 5$).

It is known that activity of Kir6, Kir3, and Kir1 members is also subject to the modulation of phosphatidylinositol-4,5-bisphosphate (PIP2) (Baukrowitz et al., 1998; Huang et al., 1998; Shyng and Nichols, 1998; Leung et al., 2000). Because of the similarity between Kir1.1 and Kir4.1, we asked if PIP2 affects baseline activity of Kir4.1 and Kir4.1–Kir5.1. Exposure to 10 $M$ PIP2 (Fluka Chemie AG) for 20–30 s enhanced baseline currents of Kir4.1–Kir5.1 by $19.9 \pm 11.5$% ($n = 5$) at pH 8.5, but had no significant effect on Kir4.1 at pH 7.5 ($-3.1 \pm 2.9$, $n = 3$). The effect of PIP2 on Kir4.1–Kir5.1 was due to a selective increase in $P_{\text{open}}$ ($29.8 \pm 13.1$, $n = 5$, at pH 7.5) without significant changes in single-channel conductance ($59.9 \pm 3.3$ pS, $n = 5$, $P > 0.05$, at pH 7.5).

Modulation of Macroscopic Currents by Intracellular Protons

The effect of pH$_i$ on macroscopic Kir currents was studied using giant patches under conditions similar to that described above. At pH 8.5, inward-rectifying currents as large as 2 nA were seen in most patches obtained from Kir4.1–Kir5.1-injected oocytes at a membrane potential of $-100$ mV. Fig. 4 illustrates modulations of the Kir4.1–Kir5.1 currents by exposures of the internal patch membrane to solutions of various pH levels. The current amplitude remained roughly the same at pH 8.0 and 8.5, started decreasing at pH 7.5, and reached almost zero at pH 7.0–6.5 (Fig. 4 A). This effect was fast, reversible, and dependent on pH levels. The relationship of the current amplitude to pH$_i$ was described using the Hill equation with pKa (pH level at a half of the maximal currents) 7.45 and the Hill coefficient (h) 2.3 ($n = 9$; Fig. 4 B). When the affected currents obtained by subtraction of the remaining currents at pH 7.5 from those at pH 8.5 were scaled to the same amplitude of the baseline currents, slopes of these two current recordings were almost identical, suggesting that the pH effect is not a voltage-dependent process (not shown). In comparison, the Kir4.1 was only inhibited at much lower pH levels with pKa 5.99 and h 2.0 (Fig. 4 B).

The pH$_i$ sensitivity of the Kir4.1–Kir5.1 was affected by PIP2. The titration curve left-shifted by 0.23 pH U in the presence of 10 $M$ PIP2 (Fig. 4 B, Table I). Similar exposure to PIP2, however, had no evident effect on the pH$_i$ sensitivity of Kir4.1 currents (Fig. 4 B, Table I).

Effects of pH$_i$ on Single Channel Properties of Kir4.1–Kir5.1

Fig. 5 shows modulations of single channel activity in a conventional inside-out patch when the internal surface of the patch was exposed to solutions with different pH levels. The Kir4.1–Kir5.1 current showed a high...
channel activity at pH 8.5 ($P_{\text{open}}$ 0.934) and pH 8.0 ($P_{\text{open}}$ 0.935). Channel activity started being inhibited at pH 7.5. This inhibition is due to the appearance of repetitive bursting activity with no detectable reduction in the current amplitude (Fig. 5). The channel was shut off at pH 6.5. The $P_{\text{open}}$ of the Kir4.1–Kir5.1 currents also can be expressed as a function of pH$_i$ with $pK_a$ 7.48 and $h$ 2.3 ($n$ = 6) (Fig. 6 A).

The inhibition of $P_{\text{open}}$ was due to a decrease in the channel mean open time and an increase in the mean closed time. At pH 7.5, single-channel Kir4.1–Kir5.1 currents had a mean open time of 25.0 ± 9.2 ms ($n$ = 4), and the mean closed time 48.6 ± 7.4 ms ($n$ = 4). These figures are significantly different from those obtained at pH 8.5 (see above, $P < 0.05$). At pH 7.5, the dwell-time histograms still showed two components of time constant in the open state ($\tau_{o1}$ = 1.3 ± 0.4 ms; $\tau_{o2}$ = 30.3 ± 8.4 ms; $n$ = 4) and three in the closed state ($\tau_{c1}$ = 0.4 ± 0.1 ms; $\tau_{c2}$ = 7.5 ± 4.3 ms; $\tau_{c3}$ = 471.9 ± 173.8 ms; $n$ = 4) (Fig. 3, C and D). Among these components, the $\tau_{o2}$ was significantly reduced, and the $\tau_{c3}$ increased at pH 7.5 in comparison with those at pH 8.5 ($P < 0.05$). The reduction in the long-lasting openings and the increase in long-lasting closures are consistent with the observed bursting activity emerging at pH 7.5.

The single-channel conductance was examined at these pH levels. Fig. 6 B shows that the single-channel conductance is fairly constant at a pH range of 7.0–8.5, indicating that protons selectively inhibit $P_{\text{open}}$ without affecting the single-channel conductance. At pH 7.0...
Channel Modulation by Protons

When channel activity was markedly inhibited, the substate conductances were still clearly seen (Fig. 3), suggesting these sublevels of conductance are independent of intracellular protons.

Critical Role of Lysine 67 in pH Sensing

To understand the molecular mechanisms underlying the pH sensitivity of the Kir4.1–Kir5.1 and Kir4.1 channels, we studied the Lys67 in Kir4.1 and Thr68 in Kir5.1 using site-directed mutagenesis. The reason for choosing these residues is that, at corresponding positions, a lysine residue (Lys80) in Kir1.1 and a threonine in Kir2.3 have been known to be critical in pH sensing of these channels (Fakler et al., 1996b; Qu et al., 1999).

After the Lys67 was substituted with methionine, a counterpart residue found in the pH-insensitive Kir2.1, we found that 15% CO₂ inhibited the mutant Kir4.1 channel by only 2.3 ± 3.9% (n = 7) (Fig. 8, A and B), and the heteromeric Kir4.1–Kir5.1 by 4.1 ± 1.3% (n = 12) (Fig. 8, C and D). These figures were not signifi-

### Table I

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Macroscopic currents were recorded using inside-out patches when the intracellular side of the membranes was exposed to solutions with various pH levels. These currents were inhibited in a concentration-dependent manner by lowering pH. The Hill equation was used to describe the inhibitions with pKa and h shown in the table. n, number of patches.

Figure 3. Dwell-time histograms of single channel Kir4.1–Kir5.1. (A and B) At pH 8.5, one active channel was recorded from an inside-out patch with equal concentrations of K⁺ on both sides of patch membrane and Vm of −80 mV. (A) Open dwell-time histogram of the Kir4.1–Kir5.1 current. Data fitting was done using four stretches of data collected from the same patch with an interval of 2 s and a total recording time of 80 s. The channel open dwell-time histogram can be fitted with two exponentials with time constants $\tau_{O1}$ 1.2 and $\tau_{O2}$ 59.9 ms. (B) Closed dwell-time histogram of this channel can also be fitted with three exponentials with time constants $\tau_{C1}$ 0.2, $\tau_{C2}$ 3.5, and $\tau_{C3}$ 224.6 ms. (C and D) Activity of the same channel ($P_{\text{open}}$) decreased by 50% at pH 7.5. (C) The open dwell-time histogram obtained from 10 stretches of data totaling 200 s is fitted using two exponentials with $\tau_{O1}$ 1.1 and $\tau_{O2}$ 41.2 ms. (D) Closed dwell-time histogram is expressed with three exponentials with $\tau_{C1}$ 0.8, $\tau_{C2}$ 3.2, and $\tau_{C3}$ 438.7 ms.
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cantly different from that obtained from the pH-insensitive Kir2.1 channels \((2.9 \pm 1.7\%, n = 5; P > 0.05)\) (Fig. 8 E). In contrast to the K67M mutation, replacement of Thr68 in Kir5.1 with alanine (T68A) had no significant effect on \(\text{CO}_2\) sensitivity of the heteromeric Kir4.1–Kir5.1 \((69.6 \pm 8.3, n = 3; P > 0.05\) in comparison with wild-type Kir4.1–Kir5.1), suggesting that the enhanced pH sensitivity in Kir4.1–Kir5.1 is not due to introducing this residue.

The effects of the Lys67 mutations on channel sensitivity to \(\text{pH}_i\) were examined in inside-out patches. Consistent with our data obtained from the whole-cell voltage clamp, the K67M mutation completely eliminated \(\text{pH}_i\) sensitivity of the homomeric Kir4.1 (Fig. 8 E). Interestingly, the heteromeric Kir4.1–Kir5.1 with the K67M mutation remained \(\text{pH}_i\) sensitive. Such \(\text{pH}_i\) sensitivity, however, was eliminated in the presence 10 \(\mu\text{M PIP}_2\) (Fig. 8 E); i.e., this mutant was only modestly inhibited at extremely acidic \(\text{pH}_i\) with \(\text{pH}\) sensitivity similar to the K67M-mutant Kir4.1. Since there are micromolar concentrations of \(\text{PIP}_2\) in oocytes (Huang et al., 1998), these results suggest that heteromeric Kir4.1–Kir5.1 with the K67M mutation loses its sensitivity to \(\text{CO}_2\) and \(\text{pH}_i\) when \(\text{PIP}_2\) is available in the cytosol.

To understand how \(\text{PIP}_2\) affects \(\text{pH}_i\) sensitivity of the Kir4.1–Kir5.1, we mutated the potential \(\text{PIP}_2\)-binding site Arg178 in Kir5.1. The Arg178 is a conserved residue found in most Kir channels, which has been shown to be a potential \(\text{PIP}_2\)-binding site in Kir6.2 (Fan and Makielski, 1997). This mutation (R178Q) completely abolished the effect of \(\text{PIP}_2\), and the mutant showed identical \(\text{pH}_i\) sensitivity with or without \(\text{PIP}_2\) in the internal solution (Fig. 9). Combined mutations of K67M in Kir4.1 and R178Q in Kir5.1 rendered a channel that showed the \(\text{pH}_i\) sensitivity similar to the Kir4.1–Kir5.1 with single K67M mutation, and its \(\text{pH}_i\) sensitivity was not influenced by \(\text{PIP}_2\) (Fig. 9). These results thus indicate that \(\text{PIP}_2\) decreases \(\text{pH}_i\) sensitivity of the hetero-
DISCUSSION

In our current studies, we have demonstrated the first K$^+$ channel that responds to either an increase or a decrease in intracellular pH from its physiological level, shown the modulation of pH sensitivity of Kir channels by PIP$_2$, and provided information about the biophysical and molecular mechanisms underlying the modulation of the heteromeric Kir4.1–Kir5.1 channels by CO$_2$ and pH.

Baseline Single-Channel Properties

Baseline single-channel properties of the homomeric Kir4.1 and heteromeric Kir4.1–Kir5.1 have not been well studied previously. The only known single-channel property as yet is the conductance. In cell-attached patches, the Kir4.1 shows a single-channel conductance of 16 pS and the Kir4.1–Kir5.1 has 40 pS (Bond et al., 1994; Pessia et al., 1996). In a separate study, we looked at single-channel properties of the Kir4.1 (Yang and Jiang, 1999). In excised inside-out patches, the Kir4.1 shows a single-channel conductance of 22 pS with a high $P_{\text{open}}$. Channel activity decreased at pH 7.5 ($P_{\text{open}}$ 0.548) and was almost completely inhibited at pH 7.0 ($P_{\text{open}}$, 0.001). Channel activity resumed to the baseline level ($P_{\text{open}}$ = 0.899) after washout. C, closure; O, opening.

![Figure 5. Concentration-dependent inhibition of single-channel activity by low pH. Single-channel currents were recorded from an inside-out patch using symmetric concentrations of K$^+$ (150 mM) on both sides of the patch. At Vm of -80 mV, one active channel was seen at pH 8.5, which had a $P_{\text{open}}$ 0.934. No detectable change in channel activity was found at pH 8.0 ($P_{\text{open}}$ 0.935). Channel activity decreased at pH 7.5 ($P_{\text{open}}$ 0.548) and was almost completely inhibited at pH 7.0 ($P_{\text{open}}$, 0.001). Channel activity resumed to the baseline level ($P_{\text{open}}$ = 0.899) after washout. C, closure; O, opening.](image)

![Figure 6. Effects of intracellular pH on $P_{\text{open}}$ and single-channel conductance. (A) At pH 7.5, the channel $P_{\text{open}}$ is only about a half of its value at pH 8.5 (average $P_{\text{open}}$ 0.846). The $P_{\text{open}}$ reaches almost its maximum level at pH 8.0, and becomes nearly zero at pH 6.5. The relationship of $P_{\text{open}}$ versus pH can be expressed with the Hill equation (solid line). The pKa (the midpoint of the channel inhibition) and h (the Hill coefficient) are pH 7.48 and 2.3 ($n$ = 6), respectively. (B) In contrast to $P_{\text{open}}$, single-channel conductance retains at ~59 pS and does not change with pH levels. Data are presented as means ± SEM.](image)
excised patches are ~30% larger than those measured in cell-attached patches, which seems to result from different K⁺ concentrations used in these two experimental conditions (150 mM in the present study vs. 90 mM by Pessia et al., 1996). At pH 7.4, the Kir4.1–Kir5.1 channels have a moderate baseline activity ($P_{\text{open}} \sim 0.4$) that increases with an increase in pHᵢ and decreases with a drop in pHᵢ, suggesting that these channels are potentially down or up regulated by either acidic or alkaline pH. At pH 8.0–8.5, when channel activity reaches its maximum, the Kir4.1–Kir5.1 channels show two open and three closed states with a mean open time of ~50 ms. Another interesting feature of these channels is the substate of conductance. These K⁺ channels show two sublevels of conductance a ~1/4 and 3/4 of the conductance of the full openings at pH 8.0–8.5. The sublevels of conductance have also been observed in outward currents of Kir2.1 (Omori et al., 1997) and inward currents of Kir2.3 (Zhu et al., 1999). In Kir2.1, the substates of conductance are related to internal Mg²⁺, while the causes for their appearance in the inward currents of Kir2.3 and the Kir4.1–Kir5.1 are not clear. Mg²⁺ is not involved since there is not Mg²⁺ in the internal solutions in both experiments on Kir2.3 (Zhu et al., 1999) and Kir4.1–Kir5.1. Although the substate conductance has been seen in several proton-gated K⁺ channels, including Kir2.3 and Kir4.1–Kir5.1, it is not found in Kir1 channels (Choe et al., 1997; McNicholas et al., 1998). Thus, the substate conductance may not be a prerequisite for the proton gating of K⁺ channels. Supporting this idea are our data showing that these substates of conductance are not affected by lowering pHᵢ in Kir4.1–Kir5.1. The presence of substate conductance as well as the relatively large conductance and moderate baseline $P_{\text{open}}$ makes the heteromeric Kir4.1–Kir5.1 clearly different from the homomeric Kir4.1 (Yang and Jiang, 1999), further supporting that they are indeed two distinct K⁺ channels (Pessia et al., 1996).

It is known that baseline channel activity of Kir channels can be modulated by PIP₂ (Baukrowitz et al., 1998; Huang et al., 1998; Shyng and Nichols, 1998; Leung et al., 2000). We also looked at the modulation of Kir4.1 and Kir4.1–Kir5.1 by PIP₂. We found that baseline Kir4.1–Kir5.1 currents were enhanced by PIP₂, which was due to an augmentation of $P_{\text{open}}$ without affecting single-channel conductance. Interestingly, PIP₂ did not
Channel Modulation by Protons

affect Kir4.1, suggesting that PIP2 interacts with Kir5.1 rather than Kir4.1. Supporting this idea are our mutagenesis data showing that the R178Q mutation completely eliminated the PIP2 effect. Thus, it is possible that PIP2 binds to the arginine residue through electrostatic charges, affecting the protein conformation and channel activity, as suggested previously (Fan and Mackielski, 1997; Huang et al., 1998).

Detection of CO2 and Intracellular Protons

Several members of the Kir4 family have been demonstrated to be pH sensitive. Shuck et al. (1997) have cloned a Kir channel from the kidney (named Kir1.2) that has a 97% identity to the brain Kir4.1. This renal K+ channel is inhibited by pH. The pH sensitivity of this Kir (pKa 6.2) is slightly higher than the brain Kir4.1. Kir4.2 cloned from the liver is also pH sensitive. It has 64% amino acid sequence homology to the brain Kir4.1 (Pearson et al., 1999). This channel is markedly inhibited by intracellular acidification produced by 50 mM HCO3−, although its pKa value is still unknown. In our previous studies, we have demonstrated that the brain Kir4.1 is sensitive to CO2 and intracellular pH (Yang and Jiang, 1999). Interestingly, we found that channel sensitivity to CO2 and pH is dramatically enhanced when the Kir4.1 is coexpressed with brain Kir5.1 (Xu et al., 2000). It is known that the Kir5.1 tends to form heteromeric channels with other Kir members, particularly with Kir4.1 (Fakler et al., 1996a; Pessia et al., 1996), in which several new biophysical properties appear. Our current data indicate that such a heteromeric expression also changes in a major way channel modulations by intracellular protons.

Mechanisms for the Proton Sensitivity of these Kir Channels

Our previous studies have indicated that a decrease in pH during CO2 exposure is the primary cause for the Kir channel inhibition (Xu et al., 2000), which is consistent with our observations in the current studies. Furthermore, our results have shown that the inhibition of
The R178Q mutant showed pHi sensitivity similar to the wild-type inside-out patches in the absence or presence of PIP2. Considering the presence of micro-anelor molar concentrations of PIP2 in an intact cell (Huang et al., 1998), our results suggest that the more realistic effect of pH on these two different single-channel properties, however, is not seen in the heteromeric Kir4.1–Kir5.1, even though it appears to have two Kir4.1 subunits in its channel protein. What causes this difference is unclear. It is possible that introducing the Kir5.1 subunit does not exist in the wild-type channel and the pH sensitivity of the heteromeric Kir4.1–Kir5.1 to detect pH fluctuations in most physiological and pathophysiological conditions.

The inhibition of Kir channels by low pH is unlikely to be caused by changes in concentrations of cytosolic soluble factors such as second messengers, polyamines, or Mg2+ since it is seen in cell-free excised patches. Also, our results do not support the idea that protein phosphorylation is responsible for the modulation of these K+ currents by intracellular protons. Several blockers of phosphatase and phosphodiesterase such as vanadate, fluoride, and pyrophosphate were used in the intracellular solution. These chemicals tend to inhibit protein dephosphorylation. In addition, there was no Mg2+ or ATP in this intracellular solution. Under such a condition, the turnover of protein phosphorylation and dephosphorylation should not occur, at least in the time frame of our low pH experiments (0.5–2.0 min). Therefore, the modulation of the Kir4.1–Kir5.1 during low pH may not be a result of protein phosphorylation.

Our studies suggest that amino acid sequences and tertiary structures in Kir channel proteins are the molecular basis underlying the modulation of these K+ channels by protons. We have found that Lys67 in the Kir4.1 is critical in the modulation. Channel sensitivity to CO2 is completely eliminated when this lysine residue is mutated to methionine or glutamine. A lysine residue found at the same position of Kir1.1 and Kir1.2 channels has been demonstrated to play a crucial role in pH sensing of these channels (Fakler et al., 1996b; McNicholas et al., 1998). Interestingly, our data have shown that this lysine residue is not only critical in pH modulation of the homomeric Kir4.1, but is also responsible for proton detection in the heteromeric Kir4.1–Kir5.1, as the K67M and K67Q mutations also totally abolish CO2 sensitivity of the Kir4.1–Kir5.1. It is known that lysine is titratable only at extremely high pH levels with pKa 10.5. Previous studies have suggested that adjacent residues to the Lys80 in Kir1.1 may be involved in reducing its pKa value to a physiological pH range (Fakler et al., 1996b; Choe et al., 1997). If this is also the case in the Kir4.1–Kir5.1, the influence of amino acid residues appears to be stronger in Kir5.1 than in Kir4.1 (Schulte et al., 1999). Such close interaction between these two subunits may underlie the high CO2/pH sensitivity of the heteromeric channels. Since PIP2 seems to favor membrane association of the intracellular protein domains (Shyng and Nichols, 1998), the interaction of Kir5.1 with Kir4.1 may be limited in the presence of PIP2, leading to a shift of the titration curve to a lower pH level. The tendency of reducing pH sensitivity persists and is even exaggerated in the K67M mutant through certain unknown processes. Despite the fact that the methionine does not exist in the wild-type channel and the pH sensitivity of the K67M-mutant Kir4.1–Kir5.1 appears to be

Interestingly, we found that the pH sensitivity of the heteromeric Kir4.1–Kir5.1 is modulated by PIP2. Channel sensitivity to intracellular protons is reduced in the presence of PIP2. Considering the presence of micromolar concentrations of PIP2 in an intact cell (Huang et al., 1998), our results suggest that the more realistic pKa level of the heteromeric Kir4.1–Kir5.1 is likely to be pH 7.2–7.3, instead of pH 7.45 as seen in cell-free excised patches. Therefore, a sharp change in channel activity appears to occur in pH 7.5–7.0, allowing the...
an artifact, our results imply that there are other proton-sensing sites in addition to Lys67. These potential titration sites seem to be located on Kir5.1 and may not be functioning in the wild-type channel. They begin to play a part only when the Lys67 is mutated and there is a lack of PIP2 in the cytosol. Since a large number of residues are involved in pH sensing (Schulte et al., 1999; Chanchevalap et al., 2000), these secondary titration sites appear to play a less important role in pH sensing of the Kir4.1–Kir5.1 channel.

We understand that this lysine residue may also be a part of the gating mechanisms in these Kir channels. With an interruption of the gating mechanism, the Kir4.1 and Kir4.1–Kir5.1 may not be able to close appropriately during hypercapnia or intracellular acidification, and thus show a marked decrease in pH sensitivity. Since the binding versus gating has been a common problem in studies of all ligand-gated ion channels (Colquhoun, 1998), the demonstration of a convergent site for proton gating in Kir4.1 and Kir4.1–Kir5.1 in our current studies may contribute to the understanding of the gating mechanisms for these K+ channels by intracellular protons.

Functional Implications
Inward-rectifier K+ channels are important players in the maintenance of plasma membrane excitability and the control of intra- and extracellular K+ ionic homeostasis. In the brainstem where the Kir4.1 and Kir5.1 channels are expressed, the inhibition of heteromeric Kir4.1–Kir5.1 channels by hypercapnia can have a major impact not only on cells expressing these channels, but also on local neuronal networks. The inhibition of these K+ channels produces depolarization and increases membrane excitability (Pineda and Aghajanian, 1997; Zhu et al., 2000), which may lead to a spread of the excitation to other brainstem neurons such as those responsible for cardio-respiratory controls. Consequently, the hypercapnic information is coupled to a corresponding change in excitability of the cardio-respiratory system (von Euler, 1986). Thus, expression of these Kir channels in brainstem neurons that have direct or indirect connections with cardio-respiratory neuronal networks may enable these cells as well as the cardio-respiratory system to detect PCO2 levels in the cerebral spinal fluid and blood circulation. In the kidney, several inward K+ channels are known to be modulated by CO2 and pH, with some of them showing high pH sensitivity (Schlatter et al., 1994; Zhou and Wingo, 1994). These K+ channels may play a role in the regulation of K+ and pH homeostasis (Wang et al., 1997). Since the heteromeric Kir4.1–Kir5.1 channels are highly pH sensitive and are also expressed in the kidney, they may be another potential candidate of the renal K+ channels responsible for pH sensing and regulating K+ secretion in the kidney. A common characteristic of these K+ channels, whether in brainstem neurons or in renal tubular cells, is the sensitivity to pH changes around the physiological level, by which channel activity can be modulated whenever pH is shifted from the physiological level. Therefore, the finding of a K+ channel with pKa 7.2–7.3 and the demonstration of the biophysical and molecular mechanisms underlying the modulation of these K+ channels by CO2 and pH constitute a significant step towards the understanding of their potential functions in CO2 and pH sensing in these cells.

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