Biophysical and Molecular Mechanisms Underlying the Modulation of Heteromeric Kir4.1–Kir5.1 Channels by CO_2 and pH

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abstract CO_2 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 CO_2 and pH. To understand the biophysical and molecular mechanisms underlying the modulation of these currents by CO_2 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}) $\sim$ 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 $\sim$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 (PIP_2) that enhanced baseline P_{open} and reduced channel sensitivity to intracellular protons. In the presence of 10 μM PIP_2, the Kir4.1–Kir5.1 showed a pKa value of 7.22. The effect of PIP_2, however, was not seen in homomeric Kir4.1 currents. The CO_2/pH sensitivities were related to a lysine residue in the NH_2 terminus of Kir4.1. Mutation of this residue (K67M, K67Q) completely eliminated the CO_2 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_i. Such pH sensitivity, however, disappeared in the presence of PIP_2. The effect of PIP_2 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 PIP_2, and provide information of the biophysical and molecular mechanisms underlying the Kir modulation by intracellular protons.

key words: CO_2 chemoreception • pH • phosphatidylinositol-4,5-bisphosphate • excised patch • brainstem

INTRODUCTION

Inward rectifier K^+ channels (Kir channels)[1] are the primary regulators of membrane excitability and are themselves also regulated by several intra- and extracellular 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 intra- and extracellular 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 CO_2 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 CO_2 chemoreception (Mitchell and Berger, 1975). Thus, detailed studies of these Kir channels may give rise to an understanding of a group of CO_2-sensitive molecules that are located on plasma membranes of nerve cells, detect neuronal ambient PCO_2 levels, and couple the PCO_2 fluctuation to a corresponding change in membrane excitability.

Several cloned Kir channels respond to CO_2 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)

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[1] *Abbreviations used in this paper:* Kir channel, inward rectifier K^+ channel; PIP_2, phosphatidylinositol-4,5-bisphosphate.

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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 CO_2 and pH, they may not be the optimum candidates of the CO_2-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 CO_2/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 pK or pKa $\sim$ 6.0), coexpression of Kir4.1 with the brain Kir5.1 greatly enhances CO_2 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 CO_2/pH sensitivities and brainstem-specific expression indicate that these channels are preferential candidates as CO_2-sensing molecules in brainstem neurons. To understand their potential role in CO_2 chemoreception, it is necessary to understand the biophysical and molecular mechanisms underlying the modulation of Kir4.1–Kir5.1 channels by CO_2 and pH.

Several mechanisms can underlie the modulation of the heteromeric Kir4.1–Kir5.1 by CO_2. Our previous studies have shown that Kir channel inhibition during hypercapnia is mediated by protons rather than molecular CO_2 (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 genetical techniques were used to answer these questions.

METHODS

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 ($\sim$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 $MgCl_2$, 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 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES, and 2.5 sodium pyruvate with 100 mg/liter geneticin 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 (pcDNA3.1; Invitrogen) was used to express Kir4.1 and coexpress Kir4.1 and Kir5.1 channels in the *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 $MgCl_2$, 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-2 $\times$ 1L headstage (input resistance $= 10^{11}\ \Omega$), and the other electrode (0.3–0.6 MΩ) was used for current recording connected to an HS-2 $\times$ 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 subtractions, 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 prepulses 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 nulled.

Patch-clamp experiments were performed at room temperature ($\sim$25°C) as described previously (Yang and Jiang, 1999; Zhu et al., 1999). In brief, fire-polished patch pipettes (2–4 MΩ) were made from 1.2-mm borosilicate capillary glass (Sutter Instrument Co.). Single channel currents were recorded from inside-out, outside-out, and cell-attached patches (Hamill et al., 1981). Giant inside-out patches were also employed to study macroscopic currents in a cell-free condition using recording pipettes of 0.5$\sim$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 nulled 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 K^+ 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 $MgCl_2$, 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.

CO_2 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 $MgCl_2$, and 5 HEPES, pH 7.4. At baseline, the chamber was ventilated with atmospheric air. Exposure of the oocytes to CO_2 was carried out by switching a perfusate that had been bubbled for at least 30 min with a gas mixture containing CO_2 (5, 10, or 15%) balanced with 21% O_2 and N_2, and superfused with the same gas. The high solubility of CO_2 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 CO_2, 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 NH_2 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_{open} was calculated by first measuring the time, t_j, spent at current levels corresponding to $j = 0,1,2,...n$ channels open (Yang and Jiang, 1999; Zhu et al., 1999). The P_{open} was then obtained as $P_{open} = (\sum^{n}_{j=1} t_j j)/tn$, where n is the number of channels active in the patch and t is the duration of recordings. P_{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 t test and were accepted as significant if $P \leq 0.05$.

RESULTS

Inhibition of Kir Currents by CO_2

Effects of CO_2 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% CO_2 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 CO_2 on these currents was reversible and depended on CO_2 concentrations. Evident inhibition of these Kir currents was seen with 5% CO_2. Higher concentrations of CO_2 (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% CO_2 produced a similar inhibition to 15% CO_2. 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 ± 1.4 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_{open} = 0.890 ± 0.021, n = 5), the heteromeric Kir4.1–Kir5.1 channels had a mean P_{open} of only 0.416 ± 0.095 (n = 6) at pH 7.4. When the pH

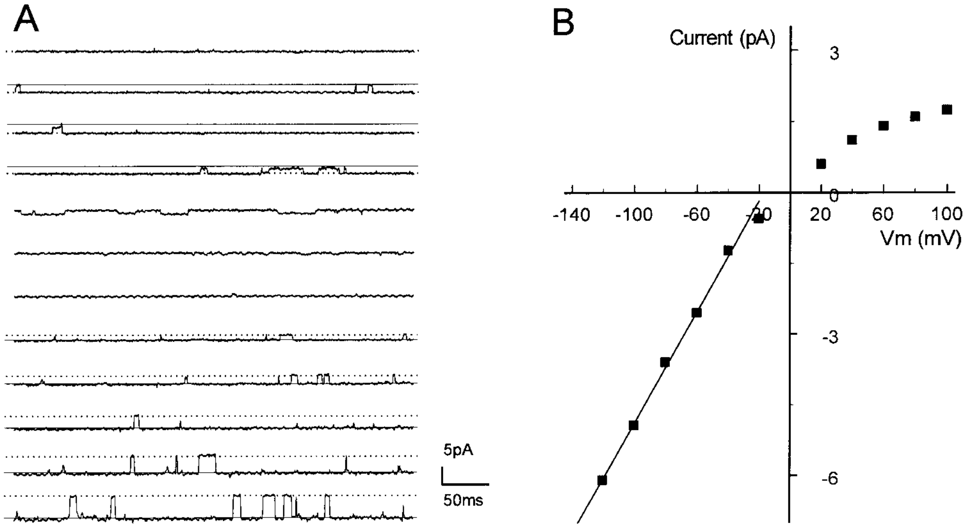

Figure 1. Single-channel conductance of Kir4.1–Kir5.1. (A) Single-channel current was recorded from an inside-out patch using symmetric concentrations of K^+ (150 mM) on both sides of the patch membrane at various membrane potentials (Vm). An active channel is seen with a clear inward rectification at hyperpolarizing Vm. Solid line, opening; dotted line, closure. (B) Single-channel conductance calculated from the channel in A is linear at Vm = −40 to −120 mV. The straight line represents a slope conductance of 59 pS.

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_{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 ± 3.6 ms (n = 4), and the mean closed time was 12.6 ± 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 (τ_{O1} = 0.9 ± 0.2 ms; τ_{O2} = 57.0 ± 3.8 ms; τ_{C1} = 0.3 ± 0.1 ms; τ_{C2} = 4.0 ± 0.3 ms; τ_{C3} = 135.5 ± 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 (PIP_2) (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 PIP_2 affects baseline activity of Kir4.1 and Kir4.1–Kir5.1. Exposure to 10 μM PIP_2 (Fluka Chemie AG) for 20–30 s enhanced baseline currents of Kir4.1–Kir5.1 by 19.9 ± 11.5% (n = 5) at pH 8.5, but had no significant effect on Kir4.1 at pH 7.5 (−3.1 ± 2.9%, n = 3). The effect of PIP_2 on Kir4.1–Kir5.1 was due to a selective increase in P_{open} (29.8 ± 13.1%, n = 5, at pH 7.5) without significant changes in single-channel conductance (59.9 ± 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 PIP_2. The titration curve left-shifted by 0.23 pH U in the presence of 10 μM PIP_2 (Fig. 4 B, Table I). Similar exposure to PIP_2, 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

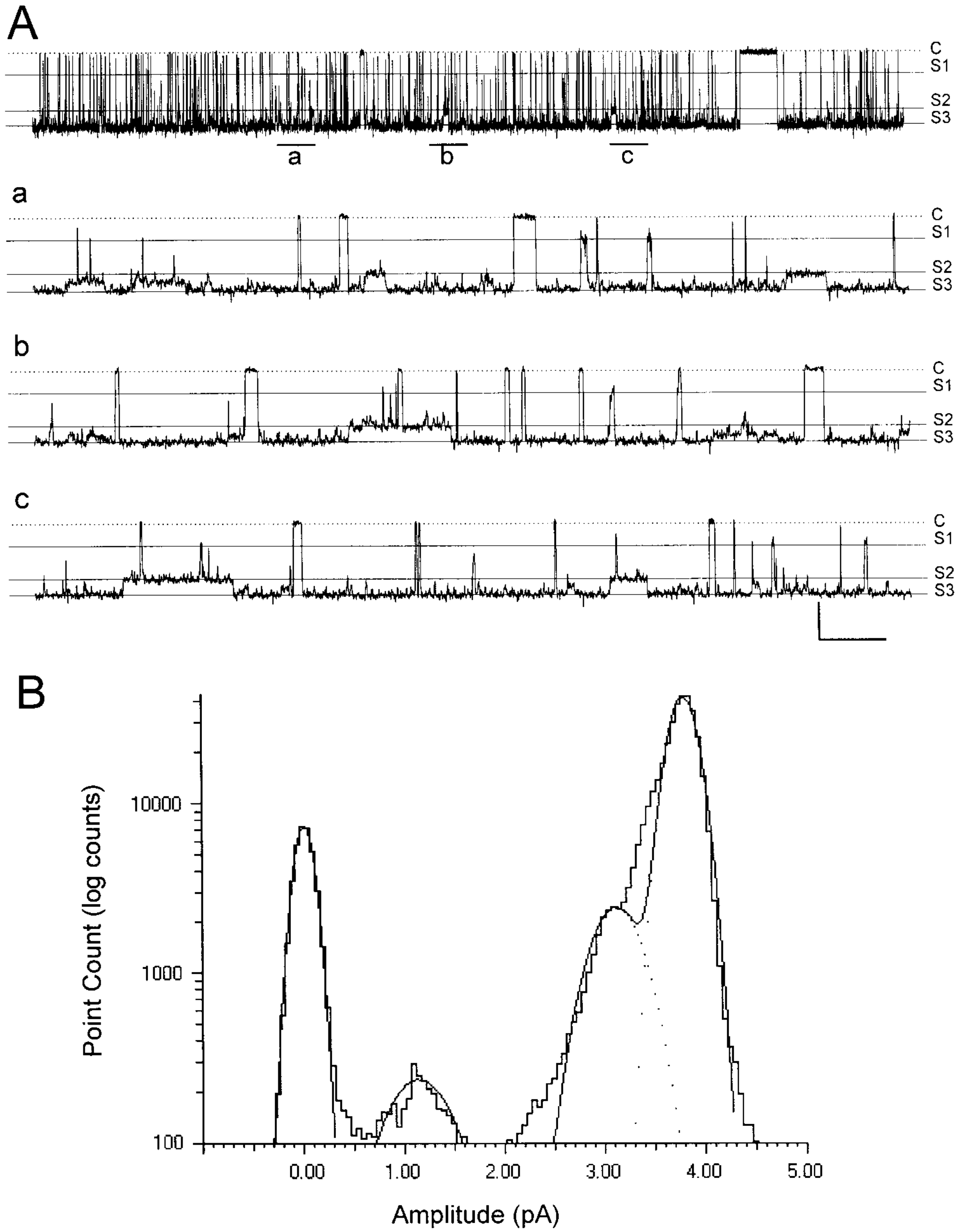

Figure 2. Single-channel activity of the Kir4.1–Kir5.1 current. (A) Single-channel activity was recorded from an inside-out patch with equal concentrations of K^+ on both sides of the patch membrane. An active channel was seen at Vm = −60 mV. At pH 8.5, this channel showed a high channel activity with P_{open} 0.915. In its long-lasting openings, brief closures can be seen that are better illustrated in extended time scales (a–c). a, b, and c are obtained from positions a, b, and c in the top trace, respectively. Also in a–c, sublevels of conductance can bee seen. Calibration: 2 s for the top trace and 200 ms for a–c; 2 pA for all. C, closure; S1, the first level of substate conductance; S2 the second level of substate conductance; S3, full opening. (B) All-point histogram of the same Kir4.1–Kir5.1 current shows channel openings at 3.78 pA at a membrane potential of −60 mV. Two sublevels of conductance are found between the closures and full openings with their peaks at ~1/4 and 3/4 of the amplitude of full openings. Data are obtained from A as a stretch recording of 20 s, displayed as a logarithmic scale in the y axis and fitted using the Gaussian distribution with each peak at 0.0, 1.14, 3.11, and 3.78 pA.

channel activity at pH 8.5 (P_{open} 0.934) and pH 8.0 (P_{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_{open} of the Kir4.1–Kir5.1 currents also can be expressed as a function of pH_i with pKa 7.48 and h 2.3 ($n = 6$) (Fig. 6 A).

The inhibition of P_{open} was due to a decrease in the channel mean open time and an increase in the mean closed time. At pH_i 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_i 7.5, the dwell-time histograms still showed two components of time constant in the open state ($\tau_{O1} = 1.3 \pm 0.4$ ms; $\tau_{O2} = 30.3 \pm 8.4$ ms; $n = 4$) and three in the closed state ($\tau_{C1} = 0.4 \pm 0.1$ ms; $\tau_{C2} = 7.5 \pm 4.3$ ms; $\tau_{C3} = 471.9 \pm 173.8$ ms; $n = 4$) (Fig. 3, C and D). Among these components, the τ_{O2} was significantly reduced, and the τ_{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_{open} without affecting the single-channel conductance. At pH 7.0

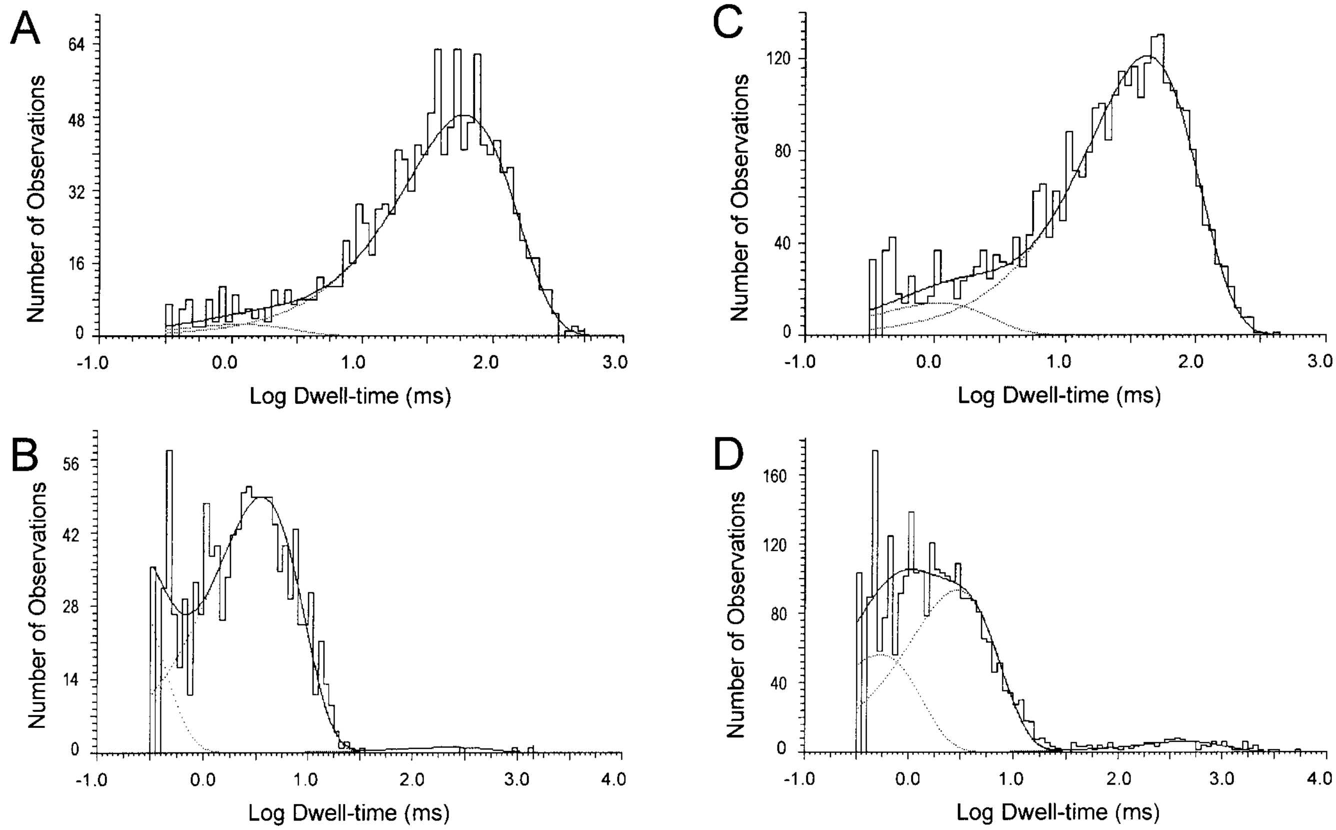

Figure 3. Dwell-time histograms of single channel Kir4.1–Kir5.1. (A and B) At pH 8.5, one active channel was recorded from an inside-out patch with equal concentrations of K^+ on both sides of patch membrane and Vm of −80 mV. (A) Open dwell-time histogram of the Kir4.1–Kir5.1 current. Data fitting was 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 τ_{O1} 1.2 and τ_{O2} 59.9 ms. (B) Closed dwell-time histogram of this channel can also be fitted with three exponentials with time constants τ_{C1} 0.2, τ_{C2} 3.5, and τ_{C3} 224.6 ms. (C and D) Activity of the same channel (P_{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 τ_{O1} 1.1 and τ_{O2} 41.2 ms. (D) Closed dwell-time histogram is expressed with three exponentials with τ_{C1} 0.8, τ_{C2} 3.2, and τ_{C3} 438.7 ms.

TABLE I

Proton Sensitivity of Kir4.1, Kir4.1–Kir4.5, and Their Mutants

Name	pKa	*h*	*n*
Kir4.1	5.99	2.0	5
Kir4.1–Kir5.1	7.45	2.3	9
Kir4.1 (PIP_2)	2.95	2.0	5
Kir4.1–Kir5.1 (PIP_2)	7.22	2.2	4
K67M_Kir4.1	4.30	0.7	5
K67M_Kir4.1–Kir5.1	6.95	2.2	5
K67M_Kir4.1–Kir5.1 (PIP_2)	4.50	0.5	5
R178Q_Kir4.1–Kir5.1	7.50	2.3	4
R178Q_Kir4.1–Kir5.1 (PIP_2)	7.50	2.3	3
K67M/R178Q_Kir4.1–Kir5.1	7.15	1.7	5
K67M/R178Q_Kir4.1–Kir5.1 (PIP_2)	7.15	1.7	4
P_{open}_Kir4.1–Kir5.1	7.48	2.3	6

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_i. The Hill equation was used to describe the inhibitions with pKa and *h* shown in the table. *n*, number of patches.

and 7.5, when channel activity was markedly inhibited, the substate conductances were still clearly seen (Fig. 7), suggesting these sublevels of conductance are independent of intracellular protons.

Critical Role of Lysine 67 in pH_i 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% CO_2 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-

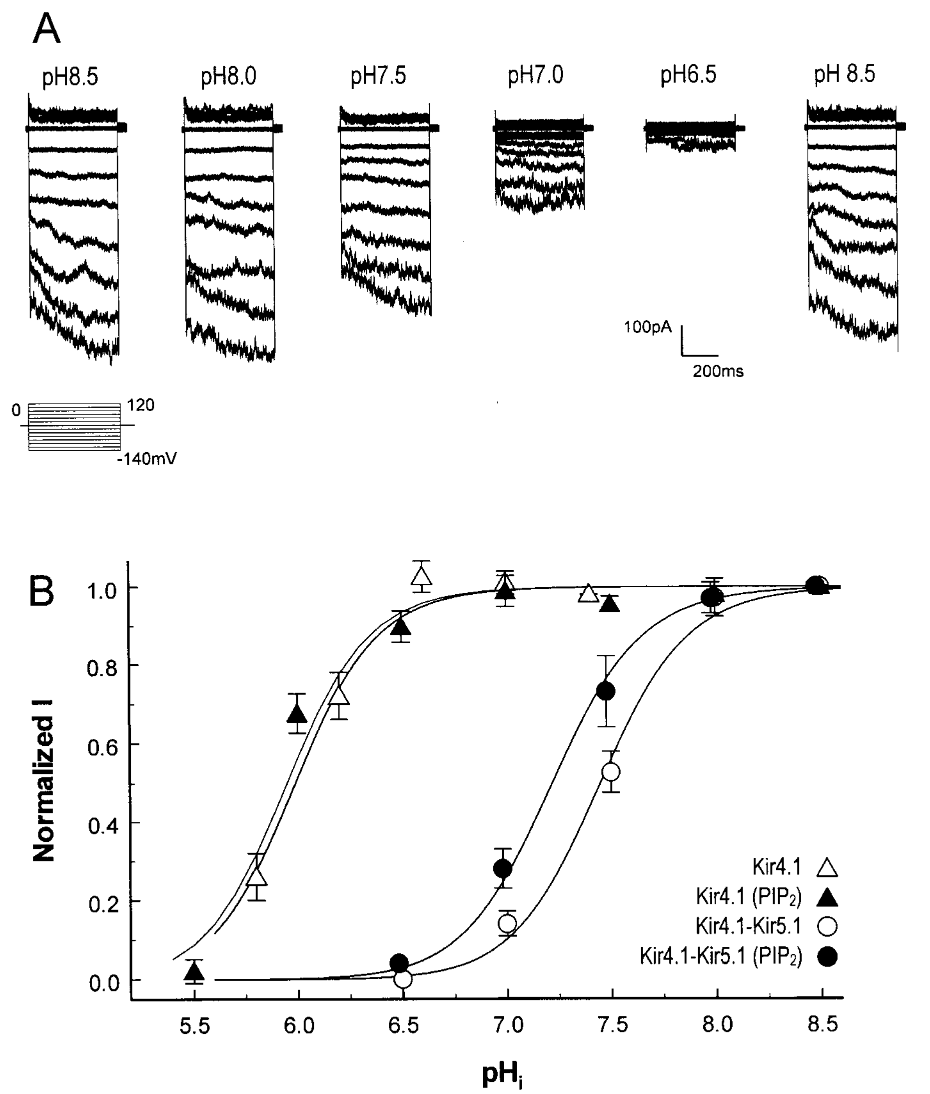

Figure 4. Effects of pH_i on macroscopic Kir4.1–Kir5.1 currents. (A) Kir4.1–Kir5.1 currents were recorded from a giant patch with equal concentrations of K^+ (150 mM) on either side of the membrane. Inward-rectifying currents were seen at pH 8.5 using command potentials from −140 to 120 mV in 20-mV increments. When pH in the internal solution was reduced to 7.5, these currents were inhibited by ~60%. Further decreases in pH_i caused strong inhibitions of these currents. Inward-rectifying currents were almost totally suppressed at pH 6.5. Washout led to a recovery of these currents to the baseline level. (B) The relationship of Kir4.1–Kir5.1 currents with pH_i. These currents are inhibited at pH 7.5, augmented at pH 8.0, and completely shut off at pH 6.5. The relationship of currents (I) to pH_i can be expressed using the Hill equation (solid line): $I = 1/[1 + (pKa/pH_i)^h]$, where pKa is the midpoint pH value for channel inhibition and *h* is the Hill coefficient. The pKa and *h* here are pH 7.45 and 2.3, respectively. In comparison, the Kir4.1 was inhibited at much lower pH levels, with pKa 5.99 and *h* 2.0. The pH sensitivity of Kir4.1–Kir5.1 but not homomeric Kir4.1 was modulated by PIP_2. In the presence of 10 μM PIP_2, its titration curve was shifted leftward by 0.22 pH U.

cantly different from that obtained from the pH-insensitive Kir2.1 channels (2.9 ± 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 CO_2 sensitivity of the heteromeric Kir4.1–Kir5.1 (69.6 ± 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 pH_i were examined in inside-out patches. Consistent with our data obtained from the whole-cell voltage clamp, the K67M mutation completely eliminated pH_i sensitivity of the homomeric Kir4.1 (Fig. 8 E). Interestingly, the heteromeric Kir4.1–Kir5.1 with the K67M mutation remained pH_i sensitive. Such pH_i sensitivity, however, was eliminated in the presence 10 μM PIP_2 (Fig. 8 E); i.e., this mutant was only modestly inhibited at extremely acidic pH_i with pH sensitivity similar to the K67M-mutant Kir4.1. Since there are micromolar concentrations of 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 CO_2 and pH_i when PIP_2 is available in the cytosol.

To understand how PIP_2 affects pH_i sensitivity of the Kir4.1–Kir5.1, we mutated the potential 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 PIP_2-binding site in Kir6.2 (Fan and Makielski, 1997). This mutation (R178Q) completely abolished the effect of PIP_2, and the mutant showed identical pH sensitivity with or without 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 pH_i sensitivity similar to the Kir4.1–Kir5.1 with single K67M mutation, and its pH sensitivity was not influenced by PIP_2 (Fig. 9). These results thus indicate that PIP_2 decreases pH_i sensitivity of the hetero-

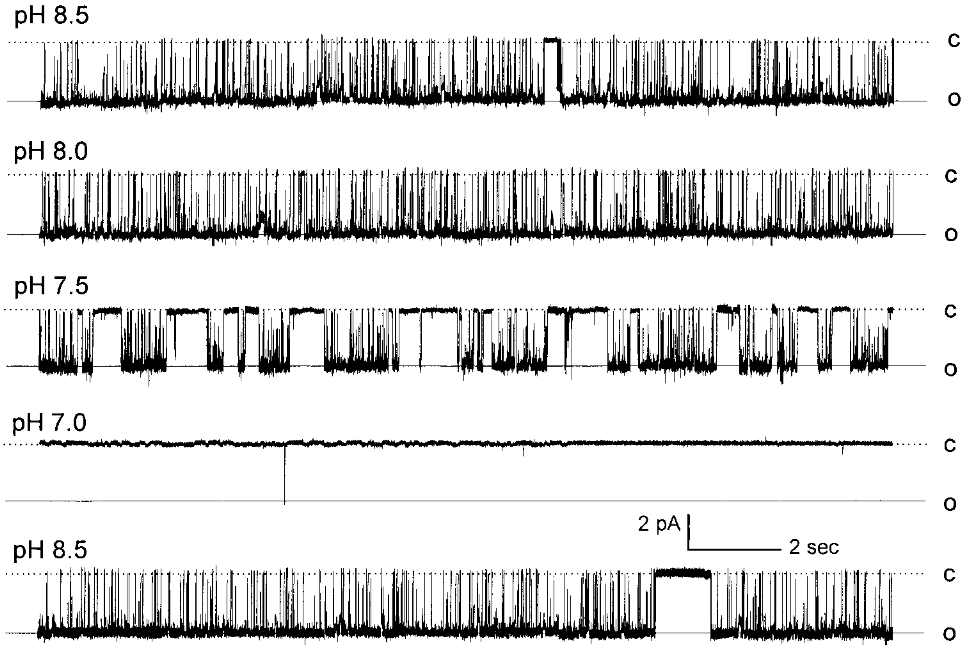

Figure 5. Concentration-dependent inhibition of single-channel activity by low pH. Single-channel currents were recorded from an inside-out patch using symmetric concentrations of K^+ (150 mM) on both sides of the patch. At Vm of −80 mV, one active channel was seen at pH_i 8.5, which had a P_{open} 0.934.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

meric Kir4.1–Kir5.1 by interacting with the Kir5.1 protein at Arg178.

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_{open} at baseline (pH 7.4). In the present study, we show that the Kir4.1–Kir5.1 has a single-channel conductance of 59 pS. Both of these figures obtained from

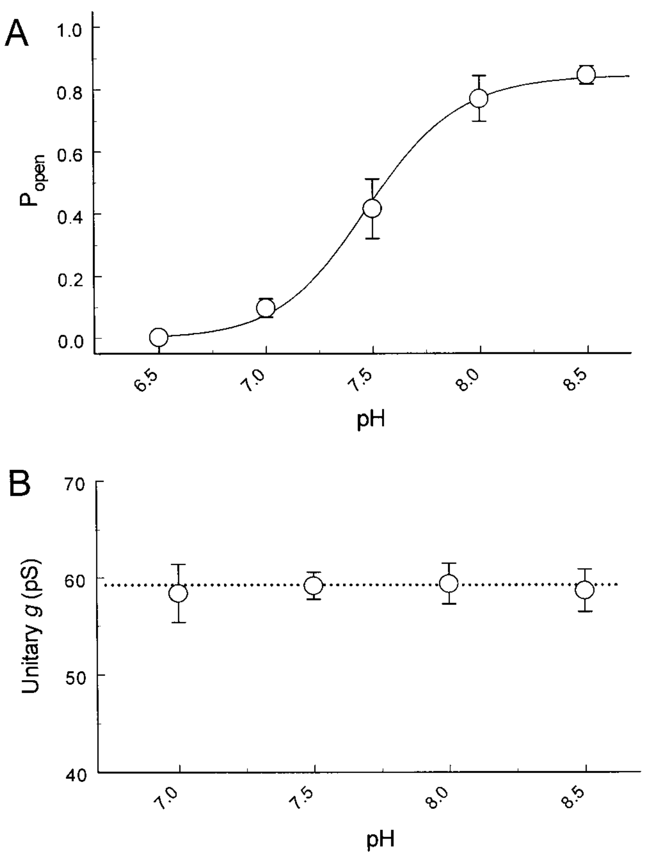

Figure 6. Effects of intracellular pH on P_{open} and single-channel conductance. (A) At pH_i 7.5, the channel P_{open} is only about a half of its value at pH_i 8.5 (average P_{open} 0.846). The P_{open} reaches almost its maximum level at pH_i 8.0, and becomes nearly zero at pH_i 6.5. The relationship of P_{open} versus pH_i 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_{open}, single-channel conductance retains at ∼59 pS and does not change with pH levels. Data are presented as means ± SEM.

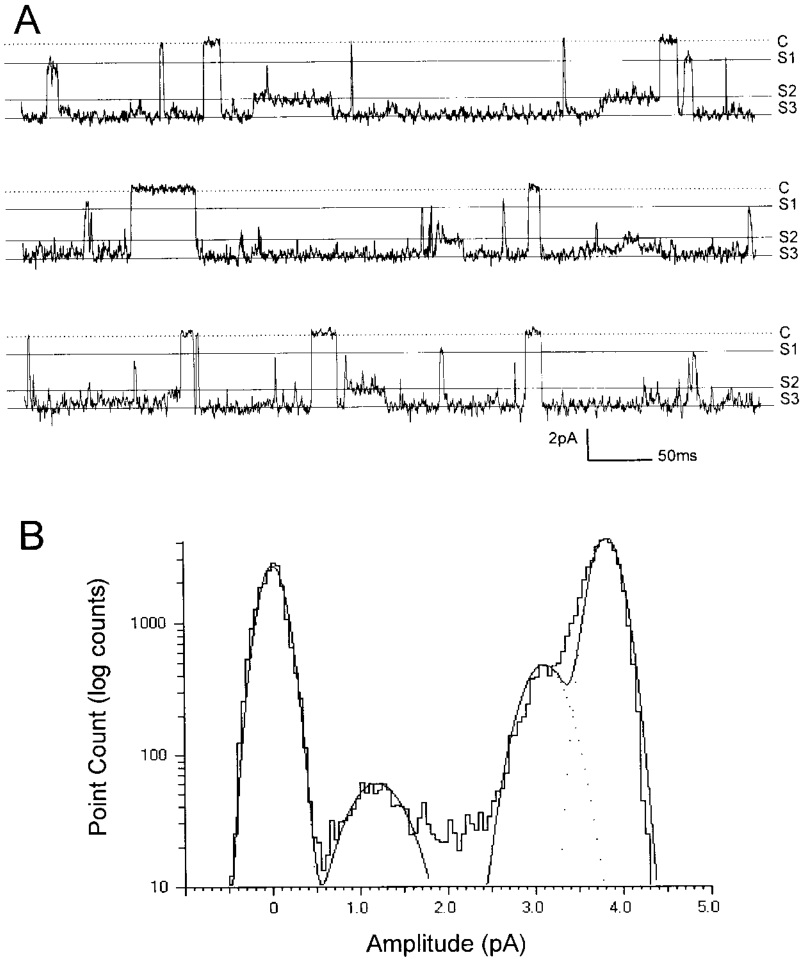

Figure 7. Lack of effect of protons on substates of conductance. (A) Single-channel Kir4.1–Kir5.1 current was recorded from the same inside-out patch as Fig. 2 at pH 7.5. Two substate conductances were still seen with peaks at ~1/4 and 3/4 of full openings, even though P_{open} was reduced 0.488 at such a pH level. C, closure; S1, substate 1; S2, substate 2. (B) All-point histogram of the current shows opening, closure, and two sublevels of conductance with each peak at 0.0, 1.18, 3.10, and 3.81 pA, respectively.

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 ($P_{open} \sim 0.4$) that increases with an increase in pH_i and decreases with a drop in pH_i, 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 Mg^{2+}, while the causes for their appearance in the inward currents of Kir2.3 and the Kir4.1–Kir5.1 are not clear. Mg^{2+} is not involved since there is not Mg^{2+} 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 pH_i in Kir4.1–Kir5.1. The presence of substate conductance as well as the relatively large conductance and moderate baseline P_{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 PIP_2 (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 PIP_2. We found that baseline Kir4.1–Kir5.1 currents were enhanced by PIP_2, which was due to an augmentation of P_{open} without affecting single-channel conductance. Interestingly, PIP_2 did not

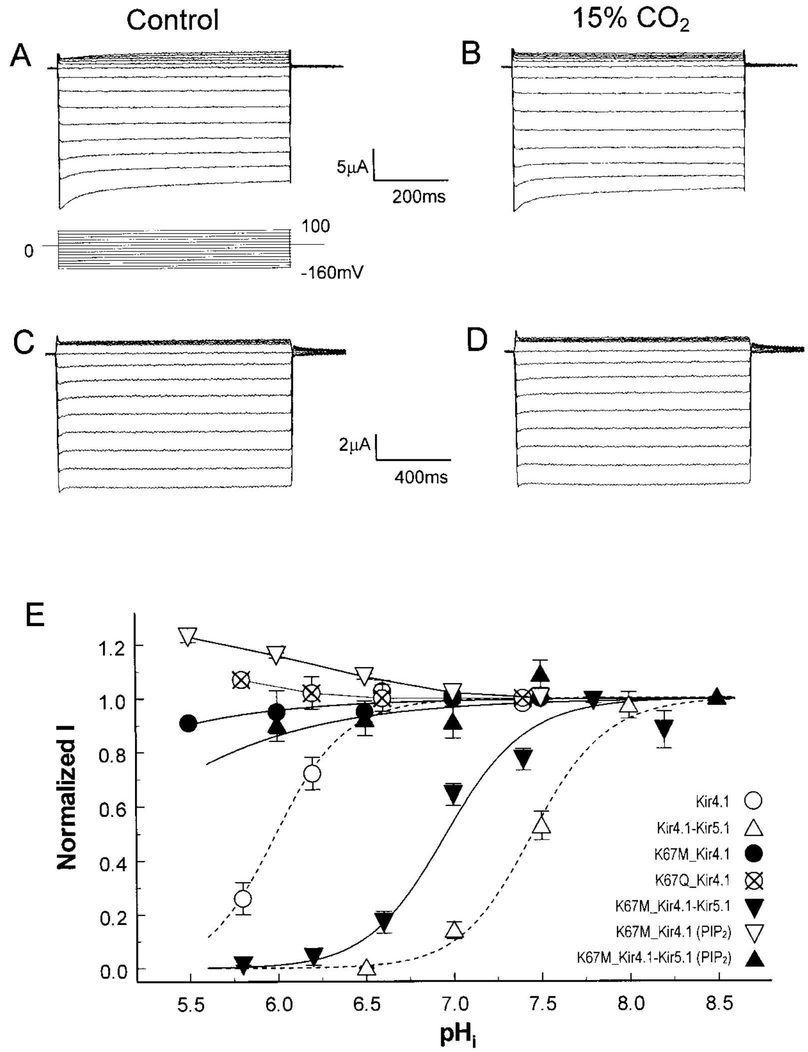

Figure 8. Effects of Lys67 mutation on CO_2 sensitivity of Kir4.1 and Kir4.1–Kir5.1 currents. (A) Whole-cell currents were recorded from oocytes. Inward-rectifier K^+ currents were seen in an oocyte that had received an injection of K67M mutant Kir4.1. (B) Although the K67M mutation did not affect the baseline currents, channel sensitivity to 15% CO_2 (5 min) was abolished. (C) The K67M Kir4.1 was created on a tandem dimer of Kir4.1–Kir5.1, the Kir4.1–Kir5.1 currents were observed 3 d after the injection. (D) These Kir4.1–Kir5.1 currents lost their hypercapnic sensitivity, as no evident change in the current amplitude was seen after 7 min exposure to 15% CO_2. (E) Titration curves of Lys67 mutations on Kir4.1 and on a tandem dimer of Kir4.1–Kir5.1. While the Lys67 mutations abolished pH_i sensitivity of Kir4.1, the Kir4.1–Kir5.1 with this mutation remained pH_i sensitive (pKa 6.95, *h* 2.2). In the presence of 10 μM PIP_2, however, Kir4.1–Kir5.1 carrying the K67M mutation lost its pH_i sensitivity.

affect Kir4.1, suggesting that PIP_2 interacts with Kir5.1 rather than Kir4.1. Supporting this idea are our mutagenesis data showing that the R178Q mutation completely eliminated the PIP_2 effect. Thus, it is possible that PIP_2 binds to the arginine residue through electrostatic charges, affecting the protein conformation and channel activity, as suggested previously (Fan and Makielski, 1997; Huang et al., 1998).

Detection of CO_2 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 HCO_3^-, although its pKa value is still unknown. In our previous studies, we have demonstrated that the brain Kir4.1 is sensitive to CO_2 and intracellular pH (Yang and Jiang, 1999). Interestingly, we found that channel sensitivity to CO_2 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 CO_2 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

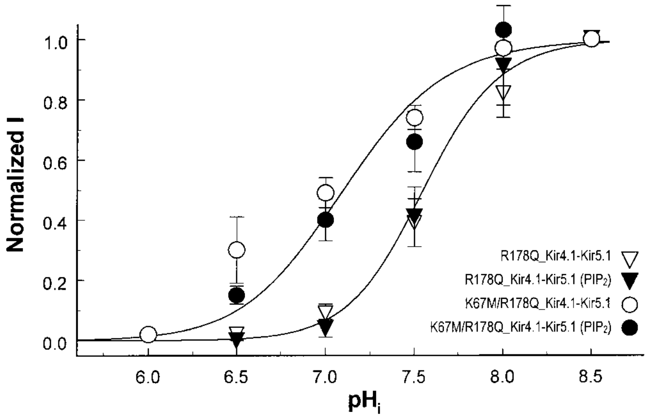

Figure 9. Mutation of Arg178 in Kir5.1 eliminated the effect of PIP_2 on channel sensitivity to pH_i. In the tandem dimer of Kir4.1–Kir5.1, site-specific mutations were made at Lys67 in Kir4.1 and Arg178 in Kir5.1. Inward-rectifying currents were then studied in inside-out patches in the absence or presence of PIP_2 (10 μM). The R178Q mutant showed pH_i sensitivity similar to the wild-type Kir4.1–Kir5.1 with pK 7.50. Its pH_i sensitivity was identical with or without PIP_2 in the internal solution. The double mutant (K67M/R178Q) showed the pH_i sensitivity (pK 7.15) similar to the Kir4.1–Kir5.1 with a single K67M mutation. Such pH sensitivity was not affected by PIP_2.

the Kir4.1–Kir5.1 by low pH is mediated by 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. The biophysical mechanisms are more like those underlying Kir1.1 channel inhibition by intracellular protons (Choe et al., 1997; McNicholas et al., 1998) than those for the Kir2.3 (Zhu et al., 1999) and the homomeric Kir4.1 inhibitions (Yang and Jiang, 1999). In the Kir4.1, we have shown that intracellular protons inhibit P_{open} and augment the single-channel conductance (Yang and Jiang, 1999). The opposite effect of pH on these two different single-channel properties, however, is not seen in the heteromeric Kir4.1–Kir5.1, even though it apparently has two Kir4.1 subunits in its channel protein. What causes this difference is unclear. It is possible that introducing the Kir5.1 subunits not only alters the architecture of the heteromeric K^+ channels, but also alters the channel modulation by intracellular ligands.

Interestingly, we found that the pH sensitivity of the heteromeric Kir4.1–Kir5.1 is modulated by PIP_2. Channel sensitivity to intracellular protons is reduced in the presence of PIP_2. Considering the presence of micromolar concentrations of PIP_2 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_i 7.5–7.0, allowing the heteromeric Kir4.1–Kir5.1 to detect pH fluctuations in most physiological and pathophysiological conditions.

The inhibition of Kir channels by low pH_i is unlikely to be caused by changes in concentrations of cytosolic soluble factors such as second messengers, polyamines, or Mg^{2+} 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 Mg^{2+} 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 CO_2 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 CO_2 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, then 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 CO_2/pH sensitivity of the heteromeric channels. Since PIP_2 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 PIP_2, leading to a shift of the titration curve to a lower pH level. The tendency of reducing pH_i 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 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 PIP_2 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 PCO_2 levels in the cerebral spinal fluid and blood circulation. In the kidney, several inward K^+ channels are known to be modulated by CO_2 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 CO_2 and pH constitute a significant step towards the understanding of their potential functions in CO_2 and pH sensing in these cells.

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