J. Membrane Biol. 190, 105–116 (2002)
DOI: 10-1007/s00232-002-1029-1

The Journal of
Membrane
Biology

Protons Activate Homomeric Kir6.2 Channels by Selective Suppression of the Long and Intermediate Closures

J. Wu, H. Xu, Z. Yang, Y. Wang, J. Mao, C. Jiang
Department of Biology, Georgia State University, 24 Peachtree Center Avenue Atlanta, GA 30302-4010, USA

Received: 1 May 2002/Revised: 15 July 2002

Abstract. The ATP-sensitive K^+ channels (K_{ATP}) play an important role in regulating membrane excitability. These channels are regulated by H^+ in addition to ATP, ADP, and phospholipids. To understand how protons affect the single-channel properties, Kir6.2ΔC36 currents were studied in excised inside-out patches. We chose to study the homomeric Kir6.2 channel with 36 amino acids deleted at the C-terminal end, as there are ADP/ATP-binding sites in the SUR subunit, which may obscure the understanding of the channel-gating process. In the absence of ATP, moderate intracellular acidosis (pH 6.8) augmented P_{open} with small suppression (by ~10%) of the single-channel conductance. The long and intermediate closures were selectively inhibited, leading to a shortening of the mean closed time without significant changes in the mean open time. Stronger acidification (< pH6.2) caused channel rundown. Although similar changes in the single-channel properties were observed in the presence of 1 mM ATP, the P_{open}-pH relationship curve was shifted by 0.17 pH units toward lower pH levels. ATP had no effect on the inhibition of single-channel conductance during acidosis. Mutation of His175 eliminated the pH effect on the single-channel kinetics, while the single-channel response to acidic pH was retained in the K185E mutant that greatly reduces the ATP sensitivity. These results indicate that Kir6.2ΔC36 channel gating by protons and ATP relies on two distinct mechanisms opposite to each other, although the pH sensitivity is modulated by ATP.

Key words: Ion channel — ATP-sensitive — pH — Gene expression — Excised patch

Correspondence to: C. Jiang; email: cjiang@gsu.edu

Introduction

The ATP-sensitive K^+ channels (K_{ATP}) regulate myocardium excitability, insulin secretion, neurotransmission and vascular tones (Quayle, Nelson & Standen, 1997; Ashcroft & Gribble, 1998; Seino et al., 2000). Activity of these channels is regulated by several cytosolic factors such as ATP, phospholipids and nucleotide diphosphates (Noma, 1983; Lederer & Nichols, 1989; Larsson et al., 1993; Inagaki et al., 1995; Fan & Makielski, 1997; Baukrowitz, 1998; Shyng & Nichols, 1998). In addition to these modulators, there is evidence indicating that the K_{ATP} channels are pH sensitive (Lederer & Nichols, 1989; Misler, Gillis & Tabcharani, 1989; Davies, 1990; Cuevas et al., 1991; Koyano et al., 1993; Fan, Tokuyama & Makielski, 1994; Proks, Takano & Ashcroft, 1994; Allard, Lazdunski & Rougier, 1995; Vivaudou & Forestier, 1995; Xu et al., 2001a). Since the pH sensitivity expands functions of these channels, detailed studies of the channel gating by protons have implications on broad physiological and pathophysiological conditions.

The gating mechanisms of K_{ATP} channels by pH are still unclear. Although the channel open-state probability (P_{open}) increases at acidic pH in the presence of ATP, it is uncertain how protons affect the single-channel kinetics in the absence of ATP. Such information is necessary for the understanding of K_{ATP} channel gating, as our recent studies have suggested that the pH sensitivity of the cloned K_{ATP} channels is seen in the absence of ATP (Xu et al., 2001a). The K_{ATP} channels run down rapidly in the absence of ATP/ADP, complicating the studies of the pH sensitivity. Indeed, reports on channel responses to pH have been rather controversial under the latter condition: acidic pH was shown to inhibit the K_{ATP} channels in cardiac myocytes, pancreatic β cells, skeletal muscles and renal tubular cells (Cuevas et al., 1991; Proks, Takano, & Ashcroft, 1994; Allard, Lazdunski & Rougier, 1995;

Mauerer, Boulpaep & Segal, 1998), stimulate it in cardiac myocytes (Fan et al., 1994), and have little effect on myocardium and frog skeletal muscles (Davies, Standen & Stanfield, 1992; Findlay, 1992). Thus, it remains to know how pH affects the K_{ATP} channels before the channel rundown takes place.

The K_{ATP} is made of the pore-forming Kir6 and the sulfonylurea receptor SUR subunit (Inagaki et al., 1995). Truncation of 24 or 36 amino acids at the C-terminal end (Kir6.2ΔC24, Kir6.2ΔC36) allows K_{ATP} channels to be expressed without the SUR subunit (Tucker et al., 1997). Using the Kir6.2ΔC36, we have recently shown that the channel activation at acidic pH relies on a histidine residue (His175) in the C terminus (Piao et al., 2001; Xu et al., 2001a,b), suggesting that there is a pH-dependent gating mechanism in these channels. To further understand the pH-dependent gating, we performed single-channel studies in excised inside-out patches. Effects of pH on the open and closed state were examined in the Kir6.2ΔC36 channel with and without ATP. The Kir6.2ΔC36 was chosen because there are ADP/ATP-binding sites in the SUR subunit, which may obscure the understanding of the channel-gating process. Our results indicate that protons activate the channel by selectively suppressing the long and intermediate closures, an effect that is exactly opposite to that of ATP. Channel gating by proton and ATP depends on distinct sites in the channel protein, although the pH sensitivity is modulated by ATP.

Materials and 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 (~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 1, Sigma Chemicals, St Louis, MO) in OR2 solution (in mM: 82 NaCl, 2 KCl, 1 $MgCl_2$, and 5 N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid or HEPES, pH 7.4) for 90 min at room temperature. After 3 washes (10 min each) of the oocytes with OR2 solution, cDNAs (20–50 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 (in mM) NaCl 96, KC1 2, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5, and sodium pyruvate 2.5 with 100 mg/l geneticin added (pH 7.4).

Mouse Kir6.2 (mBIR, Genebank #D50581) cDNA was generously provided by Dr. S. Seino at Chiba University in Japan. The Kir6.2 cDNA was removed from the PGEM-3Z vector at *EcoR* I restriction sites on each end, blunted using T4 DNA polymerase and subsequently subcloned into the *EcoR* V site in the pCDNA3.1, a eukaryotic expression vector (Invitrogen Inc., Carlsbad, CA). The truncation of last 36 amino acids was done by creating a stop codon at Leu355. The orientation of the constructs was confirmed with DNA sequencing.

Whole-cell currents were studied on the oocytes 2–4 days after injection to determine channel expression using the extracellular solution containing (in mM): 90 KC1, 3 $MgCl_2$, and 5 HEPES (pH 7.4). Our previous studies have indicated that the whole-cell Kir6.2 currents can be recorded from oocytes under physiological concentrations of ATP (Piao et al., 2001; Xu et al., 2001a). Patch experiments were undertaken in oocytes only when they showed detectable inward rectifying currents in whole-cell recordings.

Patch-clamp experiments were performed at room temperature (about 24°C) as described previously (Zhu et al., 1999; Yang et al., 2002). In brief, fire-polished patch pipettes (2–4 MΩ) were made from 1.2-mm borosilicate capillary glass (Sutter Instruments, Novato, CA). Single-channel currents were recorded from inside-out patches obtained from *Xenopus* oocytes. The oocyte vitelline membranes were mechanically removed after being exposed to hypertonic solution (400 mOsm) for 5 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 (in mM): 10 KCl, 105 potassium gluconate, 5 KF, 5 potassium pyrophosphate, 0.1 sodium vanadate, 5 EGTA, 5 glucose, and 10 HEPES (pH = 7.4). The pipette was filled with the same solution. Using this solution, we found that Kir6.2ΔC36 currents were very well maintained showing less than 10% reduction over a 15-min period of recordings at pH 7.4. This period was sufficient for all our single-channel recording protocols. Current records were low-pass filtered (2 kHz, Bessel, 4-pole filter, −3dB), digitized (20 kHz, 16-bit resolution), and stored on computer disk for later analysis (PCLAMP8, Axon Instruments).

Low-pH exposures were carried out using the same bath solutions that had been titrated to various pH levels as required. HEPES buffer was used, because of its appropriate buffering range and its impermeability to plasma membranes (Qu et al. 2000; Yang et al., 2000). For ATP exposures, pH levels were adjusted after appropriate ATP concentrations were made in each solution. Since the variation of Cl^- concentrations in the solutions was rather small, the resulting liquid junction potential was less than 1 mV according to the Henderson equation (Barry & Lynch, 1991), and was not corrected.

Single-channel conductance was measured using slope command potentials from 100 to −100 mV (150 to −150 mV in some patches). The open-state probability (P_{open}) was calculated by first measuring the time, t_j, spent at current levels corresponding to $j = 0, 1, 2, \ldots N$ channels open, based on all evident openings during the entire period of record (Zhu et al., 1999; Yang et al., 2000). The P_{open} was then obtained as $P_{open} = (\sum_J^N = 1t_{jJ})/TN$, where N is the number of channels active in the patch and T is the duration of recordings. P_{open} values were calculated from one to three stretches of data acquired using the FETCHEX software (Axon Instruments) having a total duration of 20–60 seconds. Open and closed times were measured from records in which only a single channel was active. In some patches, a second active channel appeared during acid exposures. The data from such patches were used for the open- and closed-time analysis only if the P_{open} for the second openings was not larger than 0.002. The open- and closed-time distributions were fitted using the Marquardt-LSQ method in the PSTAT6 software (Axon Instruments). Open/closed events smaller than 0.1 msec were ignored. We chose to describe the open dwell-time histograms with two exponentials and the closed dwell-time histograms with three, based on all records, although fewer exponentials could be fine for certain individual data. In some patches, the current amplitude was described using Gaussian distributions and the difference between two adjacent fitted peaks was taken as the unitary current amplitude.

The current-pH relationship was described using a sum of two Hill equations: $y = \{m/[1 + (pH/pK_1)^{h1}]\} + \{m/[1 + (pK_2/pH)^{h2}]\} - m$, where pK_1 = the pH level for midpoint channel activation, h_1 = the Hill coefficient for channel activation, pK_2 = the pH level for midpoint channel rundown, h_2 = the Hill coefficient for channel rundown, and m = 2 − percent peak activation reached at the intersection of the activation and rundown curves (Xu et al., 2001a). The activation and rundown curves were first fitted individ-

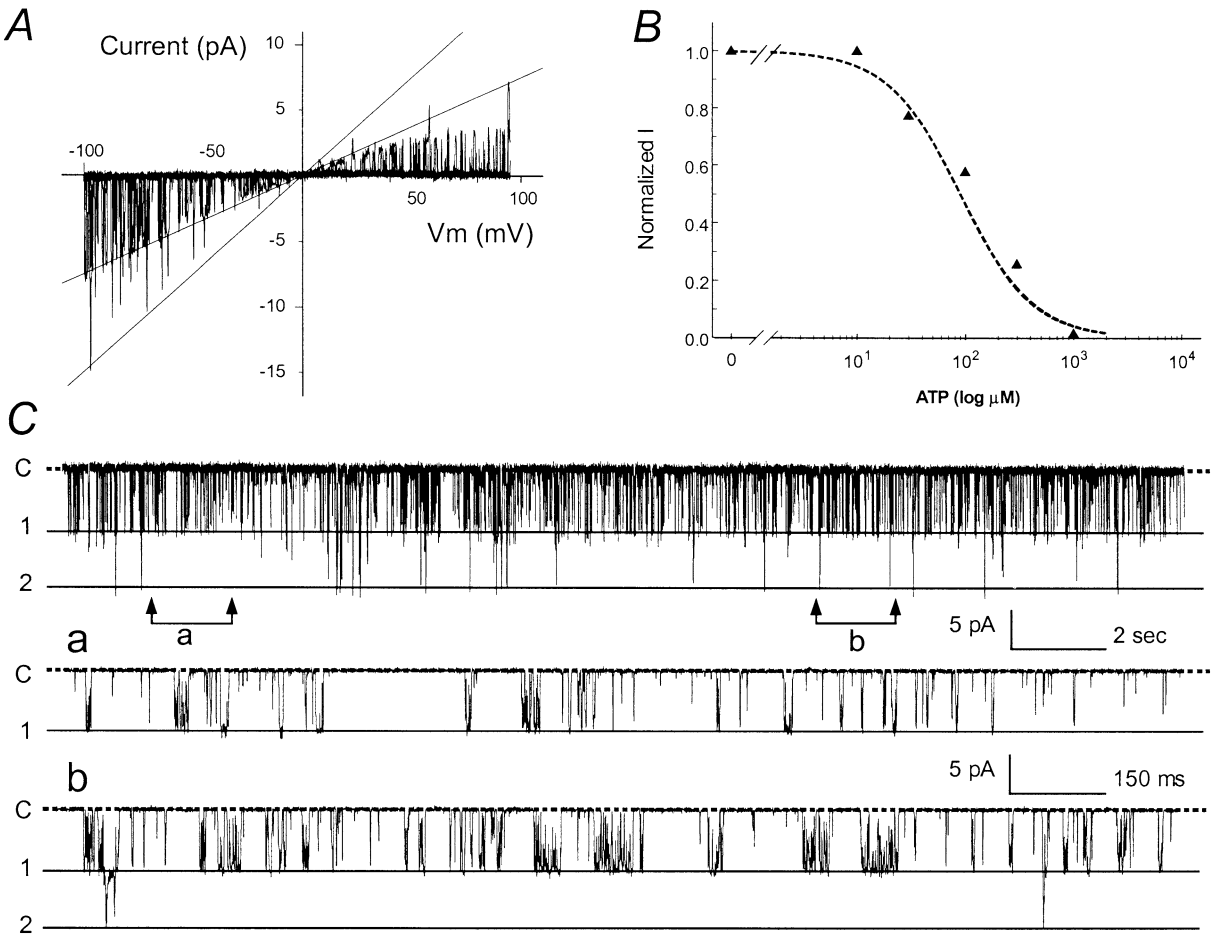

Fig. 1. Expression of Kir6.2ΔC36 currents in *Xenopus* oocyte. (*A*) Single-channel currents were recorded from an inside-out patch using symmetric concentrations of K^+ (145 mM, pH 7.4) on both sides of the patch membrane. With slope potentials from −100 to 100 mV applied to the patch membrane, two active channels are seen with a clear inward rectification at hyperpolarizing membrane potential (*Vm*). The single-channel conductance is 75 pS for both currents, as shown by the straight lines. Note that five superimposed traces are shown. (*B*) These Kir6.2ΔC36 currents were inhibited by intracellular ATP in a concentration-dependent fashion with an IC_{50} of 100 μM, as described using the Hill equation (*stippled curve*), i.e., $y = 1/(1 + ([ATP]/IC_{50})^h)$, where [ATP] = ATP concentration, h = the Hill coefficient, which is 1.3 in the equation. (*C*) Even in the absence of ATP, the Kir6.2ΔC36 currents had low baseline activity. At *Vm* of −80 mV, the channels show short periods of openings with relatively longer closures, which are better seen in the two bottom traces that are obtained from *a* and *b* in the top trace of (*C*), respectively. Abbreviation: *I*, current.

ually using a single Hill equation. The value of percent peak activation was then determined at the intersection of the activation and rundowm curves, which ranged 70–75% yielding an *m* value of 1.25–1.30. The *m* value would be 1 if the currents reached 100% peak activation when the two curves meet. The current-ATP relationship was expressed with the regular Hill equation: $y = 1/(1 + ([ATP]/IC_{50})^h)$, where [ATP] = ATP concentration, and IC_{50} = the [ATP] at midpoint channel inhibition. Data are presented as means ± SE. Differences in means were tested with the ANOVA or Student *t*-test and were accepted as significant if $P \leq 0.05$.

Results

Baseline Channel Properties

Kir6.2ΔC36 channels were studied in *Xenopus* oocytes that had received an injection of the corresponding cDNAs 2–4 days earlier. K^+ currents were recorded in the two-electrode voltage clamp using a bath solution (KD90) containing 90 mM K^+ (Piao et al., 2001; Xu et al., 2001 a,b). These currents showed clear inward rectification with an amplitude of 2.2 ± 0.4 μA (n = 8, measured at −160 mV). Exposure to 3 mM azide (Gribble et al. 1997) produced strong activation of the Kir6.2ΔC36 currents (7.6 ± 1.7 μA, n = 6). These currents were inhibited by Ba^{2+} in micromolar concentrations (*not shown*). In contrast, oocytes that received an injection of water or the expression vector alone did not display the inward rectifying currents, and the small currents were not sensitive to either azide or Ba^{2+}.

Single-channel activity was studied in inside-out patches after the expression of inwardly rectifying currents was identified in the two-electrode voltage-clamp mode. These patches were exposed to sym-

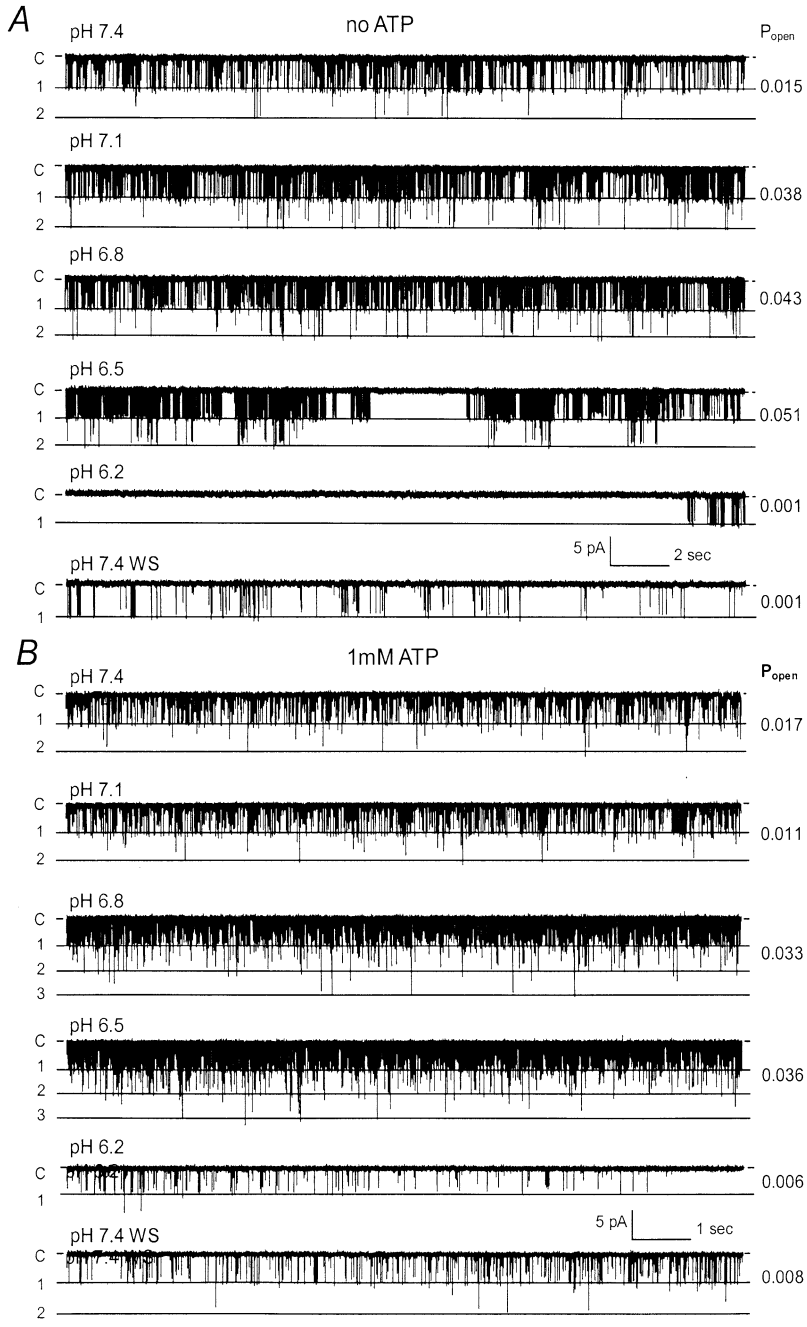

Fig. 2. (*A*) Effects of pH on single-channel activity in the absence of ATP. Single-channel currents were recorded from an inside-out patch using symmetric concentrations of K^+ on both sides of the patch membrane. At Vm of −80 mV, two active channels are seen at pH_i 7.4 with the open-state probability (P_{open}) 0.015. Channel activity increases with decreasing pH, which is evident at pH 7.1 and reaches maximum at pH 6.8–6.5 (P_{open} ~0.015). Further acidification causes marked inhibition of the channel activity at pH 6.2. The inhibition is likely related to the channel rundown, as only partial recovery can be achieved during washout. Labels on the left: *c*, closure; *1,* open level of first channel; *2*, open level of second channel. Labels on the right: P_{open} at each pH level for the particular recording period shown. P_{open} values given in legend are derived from longer records. Abbreviations: P_{open}, the open-state probability; *WS*, washout. (*B*) Augmentation of single-channel activity by acidic pH in the presence of ATP. Single-channel currents were recorded in the same condition as in *A*, but with 1 mM ATP added to the perfusates. Two active channels are seen at pH_i 7.4. Like the channel response in the absence of ATP, decreases in pH augment the channel activity. A strong inhibition of the channel activity is also revealed at pH 6.2. Channel activity did not fully recover after 3 min washout.

metric concentrations of K^+ (145 mM) on both sides of plasma membranes with command potentials from −100 to 100 mV applied to the patches through the recording pipette. Under such conditions, the Kir6.2ΔC36 currents showed single-channel conductance of 74.1 ± 1.0pS (n = 15) (Fig. 1*A*). Exposure of the internal membranes to ATP produced concentration-dependent inhibitions of the currents with an IC_{50} (ATP concentration at midpoint channel inhibition) of ~100 μM (Fig. 1*B*).

At pH 7.4 in the absence of ATP, the Kir6.2ΔC36 currents had low baseline channel activity. The channel open-state probability (P_{open}) averaged 0.025 ± 0.003 (n = 13). These single-channel Kir6.2ΔC36 currents showed short periods of openings and relatively long periods of closures (Fig. 1*C*). In patches with only one active channel, the mean open time (T_o) of these currents was 0.7 ± 0.1 msec (n = 6), and the mean closed time (T_c) 18.1 ± 2.6 msec (n = 6). The dwell-time histograms of channel openings were described with two time constants (τ_{o_1} = 0.1 ± 0.0 msec; τ_{o2} = 0.7 ± 0.1 msec, n = 6), and the closed dwell-time histograms contained three components of time constants (τ_{c1} = 0.1 ± 0.0 msec; τ_{c2} = 4.5 ± 1.2 msec; τ_{c3} = 27.9 ± 2.4 msec, n = 6) (*see* Fig. 3A_1–A_2).

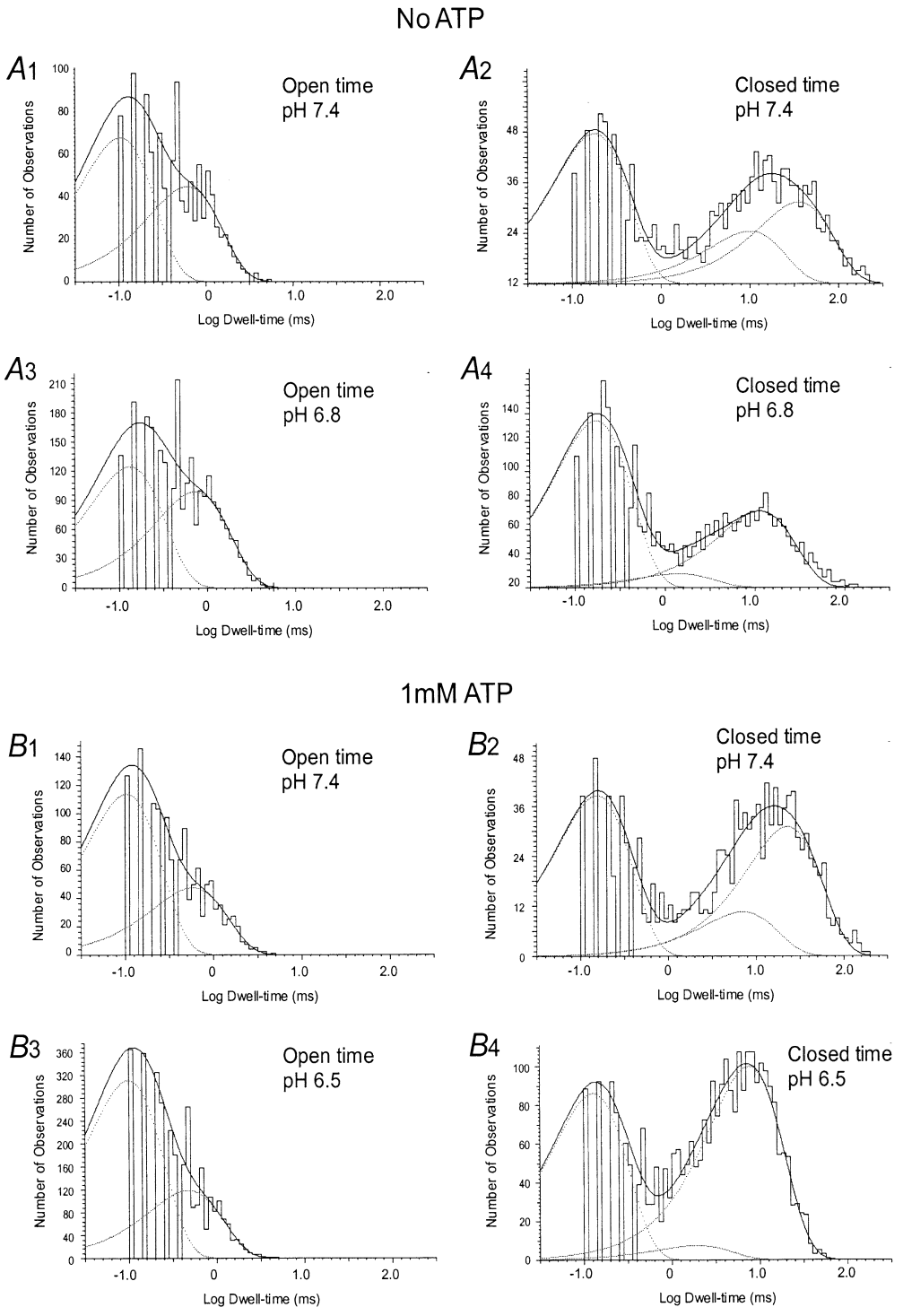

Fig. 3. Dwell-time histograms of a single Kir6.2ΔC36 channel. (*A1–A2*) In the absence of ATP, one active channel was recorded from an inside-out patch with an equal concentration of K^+ on both sides of the patch membrane and *Vm* of −80 mV. The P_{open} of the channel was 0.016 (*A*1) The channel-open dwell-time histogram can be fitted using two exponentials with time constants τ_{O1} = 0.1 msec and τ_{O2} = 0.6 msec at pH 7.4. (*A2*) The closed dwell-time histogram of this channel can be fitted with three exponentials, i.e., τ_{C1} = 0.2 msec, τ_{C2} = 9.9 msec, and τ_{C3} = 35.4 msec. (*A3*,*A4*) The open and closed dwell-time histograms of the same channel at pH 6.8 (P_{open} = 0.045). The time constants are τ_{O1} = 0.1 msec and τ_{O2} = 0.7 msec for the open dwell-time histogram (*A3*), and τ_{C1} = 0.2 msec, τ_{C2} = 2.0 msec, and τ_{C3} = 12.0 msec for the closed dwell-time histogram (*A4*). Note that only τ_{C2} and τ_{C3} are clearly different at these pH levels. (*B1–B4*) Single-channel Kir6.2ΔC36 current was studied in another patch in the presence of 1 mM ATP. (*B1*,*B2*) The channel shows single-channel properties at the baseline (P_{open} = 0.011) similar to no ATP, with τ_{O1} = 0.1 msec, τ_{O2} = 0.6 msec, τ_{C1} = 0.2 msec, τ_{C2} = 7.0 msec, and τ_{C3} = 22.8 msec. (*B3*,*B4*) At maximum activation of the channel at pH 6.5 (P_{open} = 0.036), reduction of τ_{C2} and τ_{C3} is evident (τ_{O1} = 0.1 msec, τ_{O2} = 0.5 msec, τ_{C1} = 0.1 msec, τ_{C2} = 1.7 msec, and τ_{C3} = 7.7 msec). Data were obtained from a twenty-second stretch of record in each panel.

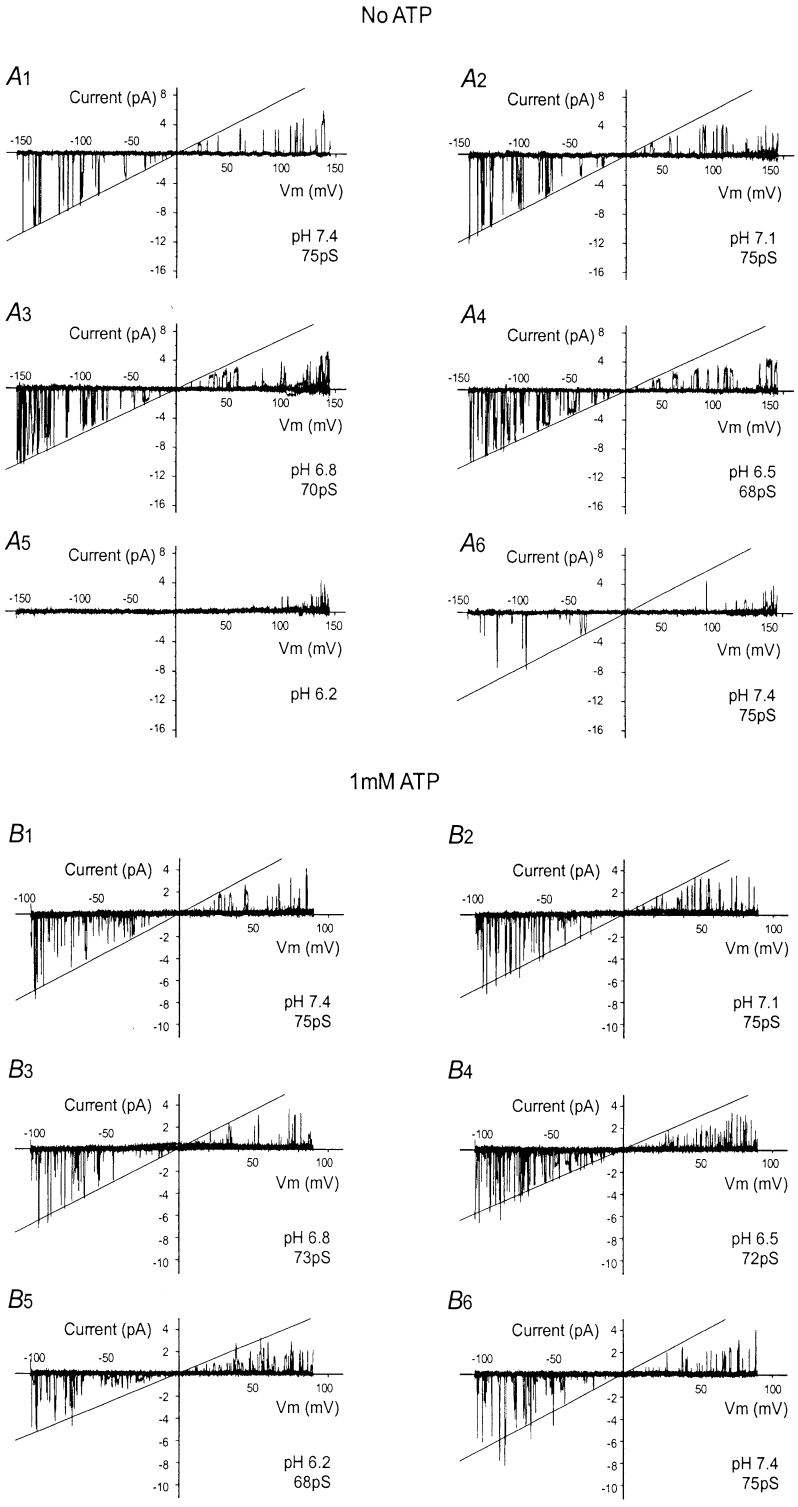

Fig. 4. Suppression of the single-channel conductance by low pH in the absence of ATP. (*A1*) Experiments were done in the same condition as Fig. 3, and the single-channel conductance was measured by fitting the inward rectifying currents with a linear equation. At pH 7.4, these currents show a slope conductance of 75 pS. (*A2–A5*) Graded decreases in pH lead to graded reduction in the single-channel conductance. Note that the pH level and conductance are shown in the forth quadrant of each panel. (*A6*) This effect is reversible, and a full recovery in the single-channel conductance is seen after washout. Five superimposed traces are shown in each panel. Effects of low pH on the single-channel conductance in the presence of ATP. (*B1–B6*) Similarly, the inhibition of the single-channel conductance occurred at low pH levels, when the perfusates contained 1mM ATP.

Effects of pH on Single-Channel Currents in the Absence of ATP

In the absence of ATP, single-channel activity was studied in conventional inside-out patches when the internal surface of the patches was exposed to solutions of various pH levels. Figure 2*A* shows the effect of pH on single-channel activity indicated by P_{open}. Noticeable increase in P_{open} was seen at pH 7.1. Maximal augmentation of the single-channel activity occurred at pH 6.8–6.5 when the P_{open} increased by 181 ± 50% (n = 7) over the baseline value. Rapid channel inhibition was observed following further acidification (Fig. 2*A*). The inhibition appeared to be

caused by the channel rundown, as channel activity showed very little recovery during washout, as reported previously (Davies et al., 1992; Koyano et al., 1993; Fan et al., 1994; Vivaudou & Forestier, 1995; Baukrowitz, et al., 1999). The augmentation of P_{open} was a result of a predominant reduction in the mean closed time (T_c = 10.3 ± 1.2 msec at pH 6.8, n = 5, $P < 0.05$ in comparison with the T_c at pH 7.4 as described above) with no significant effect on the mean open time (T_o = 0.7 ± 0.1 msec at pH 6.8, n = 5, $P > 0.05$). Consequently, the long and intermediate time constants of the closed states were selectively reduced (τ_{c1} = 0.2 ± 0.0 msec; τ_{c2} = 1.4 ± 0.3 msec; τ_{c3} = 15.0 ± 1.2 msec; n = 5; Fig. 3A_2, A_4), both of which were significantly shorter than those measured at pH 7.4 ($P < 0.01$). In contrast, the open-state time constants were not affected (τ_{o1} = 0.1 ± 0.0 msec; τ_{o2} = 0.7 ± 0.1 msec; n = 5, $P > 0.05$) (Fig. 3A_1,A_3).

Lowering pH also produced a concentration-dependent suppression of the single-channel conductance (Fig. 4A_1–A_6). This effect, however, was much smaller than that on P_{open}. At the peak level, the single-channel conductance was inhibited by 14.5 ± 3.1% (63.0 ± 1.1 pS, n = 6, measured at pH 6.2) (*see* Fig. 5*B*).

Responses to pH in the Presence of ATP

Effects of pH on the single-channel biophysical properties were also studied in the presence of 1 mM ATP in the internal solutions. Similar augmentation of P_{open} and suppression of the single-channel conductance were observed (Figs. 2*B* and 4*B*). The maximal effects occurred at pH 6.5, at which P_{open} was raised by 141 ± 20% (n = 8), a value that is not significantly different from that observed in the absence of ATP ($P > 0.05$). The augmentation of P_{open} was similarly produced by a decrease in the mean closed time (18.1 ± 0.6 msec at pH 7.4 versus 9.3 ± 2.2 msec at pH 6.5, $P < 0.01$) with no significant change in the mean open time (0.6 ± 0.1 msec at pH 7.4 versus 0.6 ± 0.1 msec at pH 6.5) (Fig. 5*C*). The dwell-time histograms showed that only τ_{c2} and τ_{c3} were significantly reduced at pH 6.5 ($P < 0.01$), i.e, τ_{o1} = 0.1 ± 0.0 msec, τ_{o2} = 0.5 ± 0.1 msec, τ_{c1} = 0.2 ± 0.0 msec, τ_{c2} = 7.6 ± 2.1 msec, and τ_{c3} = 33.0 ± 3.3 msec (pH 7.4, n = 4); τ_{o1} = 0.1 ± 0.0 msec, τ_{o2} = 0.6 ± 0.1 msec, τ_{c1} = 0.1 ± 0.0 msec, τ_{c2} = 0.7 ± 0.2 msec, and τ_{c3} = 12.5 ± 3.6 msec (pH 6.5, n = 4) (Fig. 3B_1–B_4).

The relationship of pH versus channel activity in the presence of ATP was different from that in the absence of ATP. In the presence of 1 mM ATP in the internal solution, the P_{open}-pH relationship still showed an increase at a moderate decrease in pH followed by a strong inhibition with further acidification (Fig. 5*A*). When the P_{open}-pH relationship was

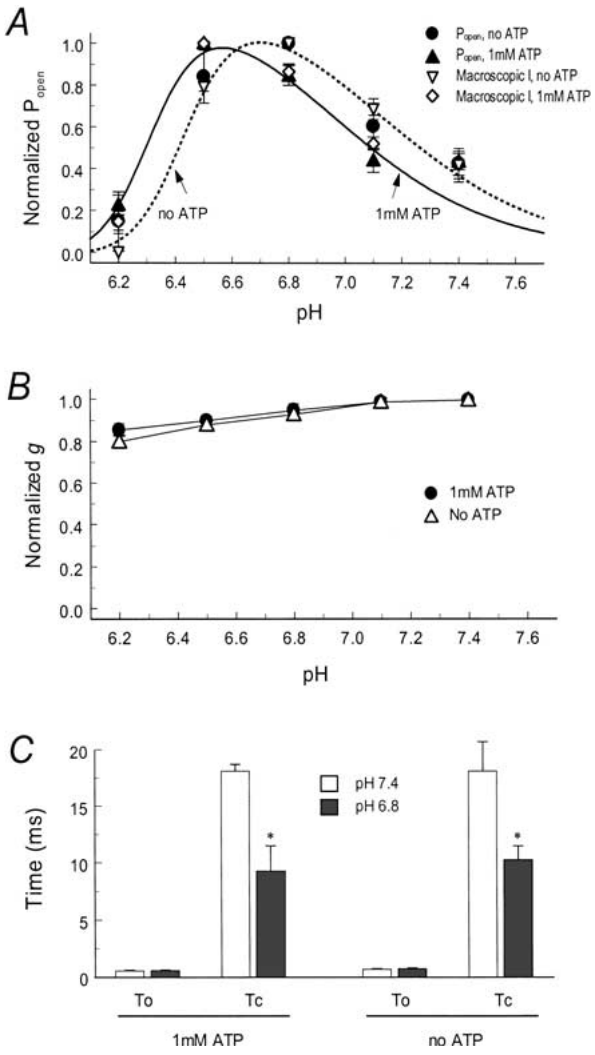

Fig. 5. The relationship of pH versus P_{open} and single-channel conductance. (*A*) At pH 7.4, the channel open-state probability (P_{open}) is less than a half of its maximum value at pH 6.5–6.8. The P_{open} is even lower at pH 6.2. The relationship of P_{open} versus pH can be expressed with a sum of two Hill equations: $y = \{1.3 / [1 + (pH / pK_1)^{h1}]\} + \{1.3/[1 + (pK_2/pH)^{h2}]\} - 1.3$. In the absence of ATP (*dashed line*), the P_{open}-pH relationship (normalized P_{open}) is fitted with the equation in which pK_1 = 7.13, h_1 = 1.6, pK_2 = 6.43, and h_2 = 4.7 (n = 7). Such a fitting can be applied to the macroscopic Kir6.2ΔC36 currents (macroscopic I) recorded from giant inside-out patches (n = 4), in which multiple active channels were recorded. Although the P_{open}-pH (n = 8) and current-pH (n = 4) relationship obtained in the presence of 1 mM ATP (*solid line*) has a similar shape as that without ATP, ATP shifts the curve toward a lower pH level (pK_1 = 6.96, h_1 = 1.6, pK_2 = 6.31, and h_2 = 4.7). (*B*) In contrast to the biphasic response of P_{open}, single-channel conductance (g) shows only inhibition, with or without ATP. (*C*) The enhancement of P_{open} at pH 6.8 is a result of selective suppression of the mean closed time (T_c) without significant changes in the mean open time (To). This was observed in the presence and absence of ATP in the internal solutions. Note that no comparison should be made between groups, since data were selected from different patches that showed only one active channel at these two pH levels. * $P < 0.05$ for data within a group. Data are presented as means ± S.E.

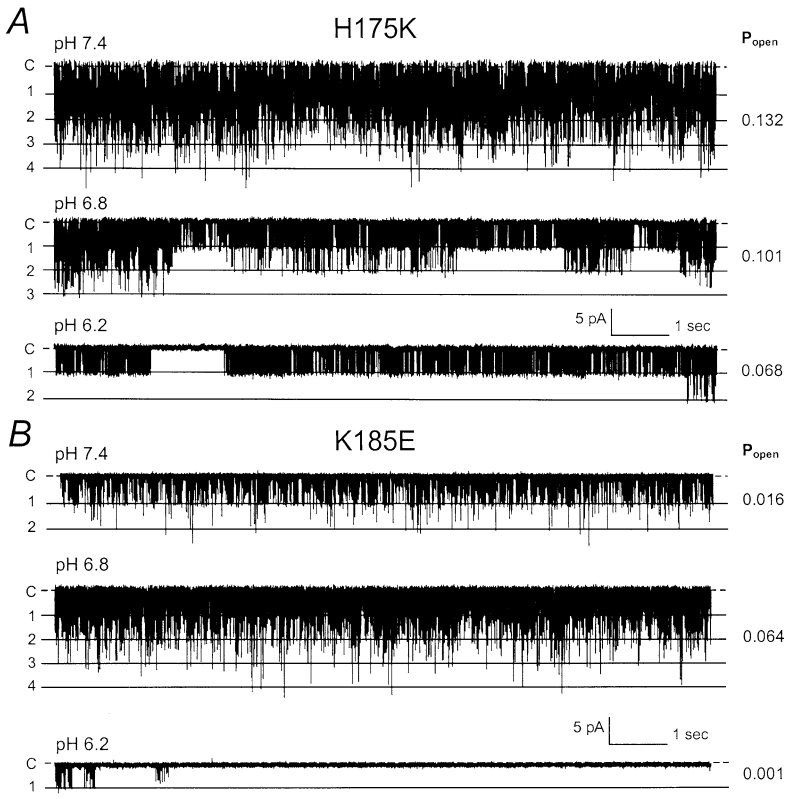

Fig. 6. Single-channel currents recorded from H175K and K185E. The experimental condition was the same as in Fig. 3, with no ATP in the perfusates. (*A*) The H175K mutant shows a high baseline (pH 7.4) activity. The P_{open} decreases at pH 6.8 and pH 6.2. Note that there is no enhancement of channel activity at acidic pH levels. (*B*) The K185E mutant, on the other hand, shows a pH response similar to the wt Kir6.2ΔC36.

plotted using a double Hill equation (*see* Methods), we found that ATP shifted the P_{open}-pH curve by 0.17 pH units toward a lower pH level with pK_1 6.96, h_1 1.6 (for channel activation), pK_2 6.31 and h_2 4.7 (for channel inhibition), in comparison to pK_1 7.13, h_1 1.6, pK_2 6.43, and h_2 4.7 in the absence of ATP (Fig. 5*A*). The relationship of pH with channel activity was also studied in macroscopic Kir6.2ΔC36 currents recorded from giant inside-out patches. The current-pH curve was highly similar to that for the P_{open}, which basically can be fitted using the same set of parameters (Fig. 5*A*). Thus, these results indicate that protons produce a biphasic response in channel activity, and ATP causes a parallel shift of the P_{open}-pH relationship to lower pH levels.

Figure 5*B* shows the relationship of pH versus the single-channel conductance with or without ATP. In the presence of 1 mM ATP, the single-channel conductance was suppressed by 12 ± 2% at pH 6.5 (n = 7), which was similar to that without ATP ($P > 0.05$). Thus, these results suggest that ATP reduces the pH sensitivity of Kir6.2 channels by affecting selectively P_{open} but not the conductance.

Dissociation of the pH Sensitivity from ATP Sensitivity

There are two possible explanations for the modulation of pH sensitivity by ATP: 1) these two K_{ATP} regulators compete for the same binding sites; 2) they bind to two different sites, with the bindings affected by each other. These possibilities can be examined by mutating single amino-acid residues that specifically affect the channel sensitivity to one of the regulators. Our recent studies have shown that the pH-dependent activation of Kir6.2 is completely eliminated by mutation of His175 (Xu et al., 2001a,b). Also, mutation of Lys185 has been previously reported to reduce greatly the ATP sensitivity (Reimann et al., 1999). Thereby, we studied the effect of pH on single-channel activity using the H175K and K185E mutants. Single-channel currents of the H175K mutant showed a baseline P_{open} of 0.162 ± 0.026 (n = 8 at pH 7.4), which was significantly higher than that of the wt Kir6.2 ($P < 0.001$). The P_{open} did not show any increase in a pH range of 7.4–6.2. Indeed, the P_{open} was suppressed by ~40% at pH 6.5–6.8 (Figs. 6*A*, *see* also 8*A*). The dwell-time histograms showed that the τ_{c2} and τ_{c3} were augmented at acidic pH, instead of the inhibition seen in the Kir6.2ΔC36 (Fig. 7A_1–A_4).

The K185E mutant showed pH sensitivity almost identical to that of the Kir6.2ΔC36 (Figs. 6*B*, 8*A*), although its ATP sensitivity was greatly diminished (Reimann et al., 1999). Such pH sensitivity remained the same in the presence of 1 mM ATP. The effect of pH on the K185E mutant was also mediated by selective inhibition of the long and intermediate closures as in Kir6.2ΔC36 (Fig. 7B_1–B_4). Thus, the

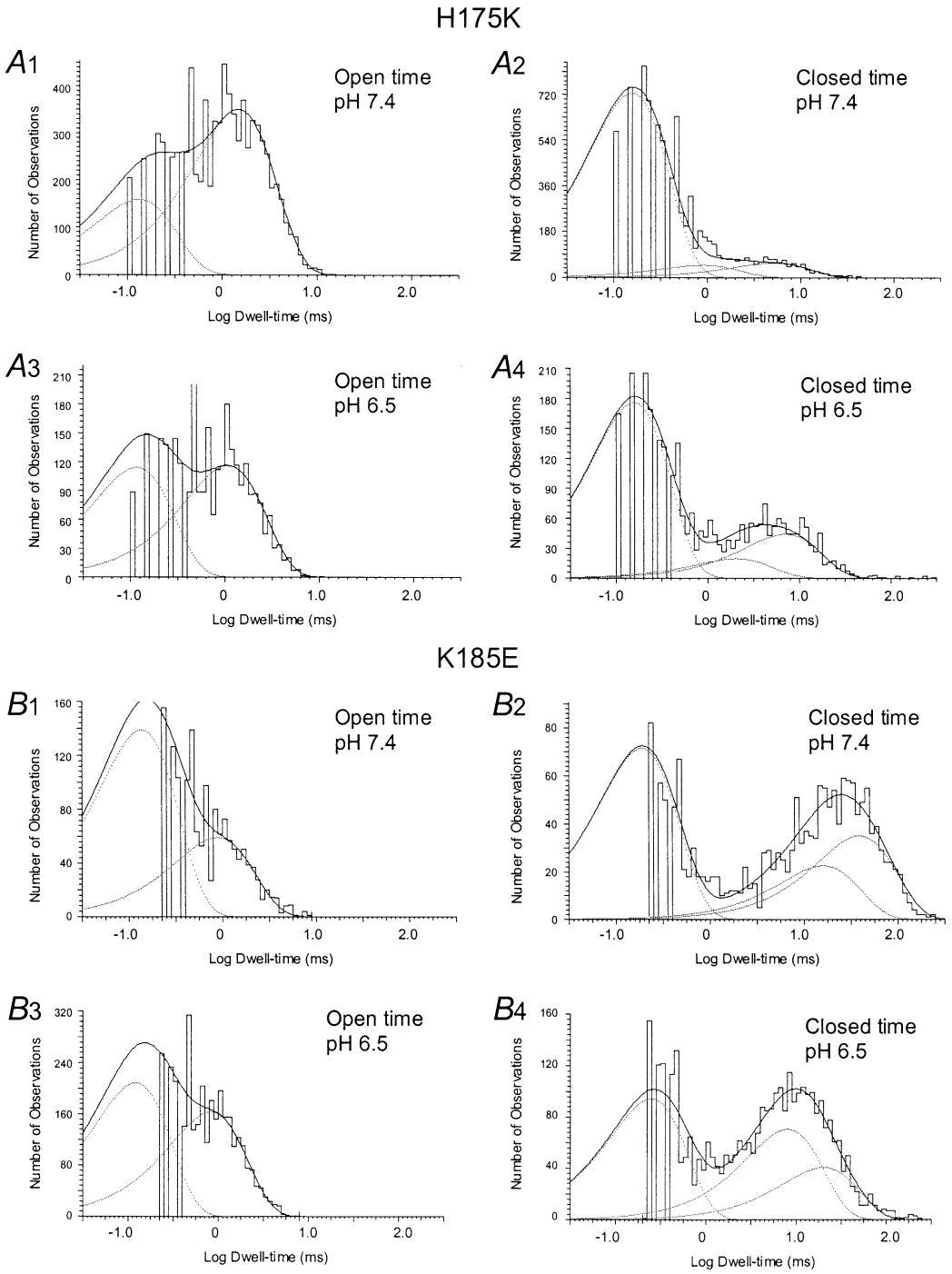

Fig. 7. Effects of H175K and K185E mutations on single-channel kinetics in the absence of ATP. (*A1–A4*) Dwell-time histograms of the H175K mutant at pH7.4 and 6.5. At pH 7.4 (P_{open} = 0.231), the open time constants are τ_{O1} = 0.1 msec and τ_{O2} = 1.5 msec (*A1*), and the closed time constants are τ_{C1} = 0.2 msec, τ_{C2} = 0.8 msec, and τ_{C3} = 5.1 msec (*A2*) The time constants are τ_{O1} = 0.1 msec and τ_{O2} = 1.1 msec for the open dwell-time histogram (*A3*), and τ_{C1} = 0.2 msec, τ_{C2} = 1.9 msec, and τ_{C3} = 7.2 msec for the closed dwell-time histogram (*A4*) at pH 6.5 (P_{open} = 0.148). (*B1–B4*) In contrast to the H175K mutant, the K185E responded to pH almost identically to the Kir6.2ΔC36 (*B1*,*B2*). At pH 7.4 (P_{open} = 0.016), the open/closed time constants are τ_{O1} = 0.1 msec, τ_{O2} = 0.8 msec, τ_{C1} = 0.3 msec, τ_{C2} = 16.6 msec, τ_{C3} = 39.2 msec. (*B3*,*B4*) At maximum activation of the channel at pH 6.5 (P_{open} = 0.035), evident reductions of τ_{C2} and τ_{C3} are also seen (τ_{O1} = 0.1 msec, τ_{O2} = 0.8 msec, τ_{C1} = 0.3 msec, τ_{C2} = 8.3 msec, and τ_{C3} = 20.1 msec). Data were obtained from two twenty-second stretches of records in each panel.

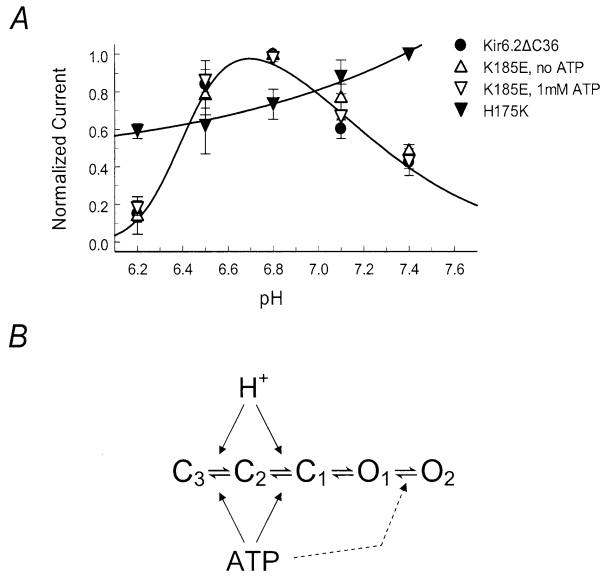

Fig. 8. (*A*) The current-pH relationship of the Kir6.2ΔC36 and its mutants. The H175K mutation eliminates the channel activation by acidic pH, while the current-pH relationship of K185E mutant in the absence and presence of 1 mM ATP remains roughly the same as that of the Kir6.2ΔC36. The relationship with pH is expressed with a sum of two Hill equations as shown in Fig. 5*A* with $pK_1 = 7.14$, $h_1 = 1.5$, $pK_2 = 6.40$, and $h_2 = 4.1$ ($n = 5$ for K185E, $n = 6$ for Kir6.2ΔC36). (B) Schematic model of the K_{ATP} gating by proton and ATP. Based on the open- and closed-time distributions from the present study, we propose a model with three closed states and two open states to describe channel activation at moderate pH levels. H^+ enhances channel activity by facilitating the transition from longer to shorter periods of closures leading to a shortening of the closed states. In contrast, ATP inhibits the channel by maintaining the long and intermediate closed states with potential inhibition to the open state, according to previous reports (Drain, Li & Wang, 1998; Trapp et al., 1998; Babenko, Gonzalez & Bryan 1999; Enkvetchakul et al., 2000; Markworth, Schwanstecher & Schwanstecher, 2000). Thus, major effects of these two K_{ATP} regulators are opposite on the control of the closed states. Note that channel rundown is not shown, which is produced by severe intracellular acidosis and can be attenuated by ATP.

K185E mutation has no effect on the pH-dependent changes in the single-channel kinetics although its ATP sensitivity is largely lost. These results therefore suggest that ATP and proton are likely to bind to distinct sites in the channel protein.

Discussion

The present study presents the first evidence that the pH-dependent activation of the Kir6.2ΔC36 channel is due to selective suppression of the long and intermediate closures, which is seen in the presence and absence of ATP. Although the pH sensitivity decreases in the presence of ATP, these two K_{ATP} channel regulators appear to act on their own distinct binding sites in the channel protein.

The Kir6.2 shares several similarities with the β cell-endogenous K_{ATP} channels. The single-channel Kir6.2 currents have a very low baseline activity with a single-channel conductance of 60–80 pS (Inagaki et al., 1995; Tuckor et al., 1997; Repunte et al., 1999). The channels are inhibited by intracellular ATP with an IC_{50} of ~100 μM, indicating that the Kir6.2 channels are properly expressed in the *Xenopus* oocytes.

Another important property of the K_{ATP}, is the pH sensitivity. In the presence of ATP, low pH (pH 6.3–7.2) enhances K_{ATP} activity in several tissues and cell types (Davies et al., 1992; Koyano, 1993; Fan et al., 1994; Proks et al., 1994; Allard et al., 1995; Vivaudou & Forestier, 1995). Studies in the absence of ATP, however, showed that pH 5.7–6.8 had inconsistent effects on channel activity (Davies, 1990; Davies et al., 1992; Findlay, 1992; Koyano, 1993; Fan et al., 1994; Proks et al., 1994; Allard et al., 1995; Vivaudou & Forestier, 1995). The pH sensitivity has been studied in the cloned K_{ATP}. Baukrowitz et al. (1999) have shown that the outward currents of Kir6.2 increase with low pH_i, resulting from a pH-dependent blockade of the channels by polyamines. Interestingly, they did not show any major changes in the inwardly rectifying currents, whereas another study showed that the inward rectifying currents were inhibited at acidic pH (Markworth et al., 2000).

We believe that inconsistency in the pH sensitivity of the cloned and cell-endogenous K_{ATP} may result from the biphasic response of these channels to pH. It is very likely that the channels are stimulated by a brief exposure to moderate acidification but inhibited with a longer exposure to stronger acidosis, as shown in the present study. In the presence of ATP, the channel rundown becomes less severe (Quayle, Nelson & Standen, 1997; Ashcroft & Gribble, 1998). Therefore, the likelihood to see channel activation increases. A similar biphasic response has been observed in the cell-endogenous K_{ATP} channels (Koyano et al., 1993; Fan et al., 1994), further indicating the consistence of the pH sensitivity between cloned and cell-endogenous K_{ATP} channels.

Our detailed studies of the pH-dependent Kir6 activation have shown that the increase in the macroscopic Kir6.2 currents recorded from giant inside-out patches and whole-cell oocytes is produced by changes in specific single-channel properties. We have found that the P_{open} increases with a moderate acidification, which is a result of a decrease in the mean closed time without a change in the mean open time. Our single-channel kinetic analyses reveal that the long and intermediate time constants of the closed states are selectively suppressed, making the closed states less stable in an acidic environment (Fig. 8*B*). Although the single-channel conductance moderately decreases in

the meantime, the overall macroscopic channel activity rises with low pH, as we have previously shown in giant patches and whole-cell recordings (Piao et al., 2001; Xu et al., 2001a,b). These effects of protons are independent of ATP and other cytosolic soluble factors, because they either are absent or are vastly diluted in the excised patches. Therefore, these observations are generally consistent with previous studies on the cell-endogenous K_{ATP} channels (Fan et al., 1994; Vivaudou & Forestier, 1995).

Although the pH sensing is independent of ATP, ATP can dramatically change the pH sensitivity of K_{ATP} channels. Vivaudou & Forestier (1995) have shown that the pK value for K_{ATP} activation shifts from pH 6.25 (with 30 μM ATP) to pH 5.30 (with 1 mM ATP) in frog skeletal muscles, a tendency that is consistent with the Kir6.2. Since such low pK values are not seen in Kir6.2ΔC36, it is possible that there are channel species sensitive to considerably low pH levels in the frog skeletal muscles. In addition to channel activation, ATP can attenuate the K_{ATP} rundown in several native tissues (Koyano et al., 1993; Fan et al., 1994). This is consistent with the leftward shift in the current-pH relationship curve shown in the present study, which we speculate to be due to alleviation of channel rundown by ATP.

The ATP sensitivity of the K_{ATP} channels is affected by pH, as has been observed in several cell-endogenous K_{ATP} channels. The IC_{50} ATP concentration is doubled from 17–25 μM at pH 7.2 to 33–50 μM at pH 6.4 (Lederer & Nichols, 1989; Koyano et al., 1993; Fan et al., 1994.). A larger increase in IC_{50} has been reported with lower pH levels (Davies et al., 1992; Vivaudou & Forestier, 1995). It is worth noting that some of the differences between the present study and previous ones may be related to the SUR subunit. It is possible that SUR subunits possess proton-sensing residues, protonation of which may affect activity or the wild-type K_{ATP} channels.

How do ATP and proton interact in regulating channel activity? Results from the present study on the Kir6.2ΔC36 and those previously done on the cell-endogenous K_{ATP} indicate that the change in pH sensitivity is a result of a parallel shift of the current-pH relationship curve, as no major change in the Hill coefficient has been found in either the cloned or the cell-endogenous K_{ATP} channels (Vivaudou & Forestier, 1995). The parallel shift of the pH sensitivity in the presence of ATP is not due to competition of protons with ATP for the same binding site. Instead, they both may have their own binding sites in the channel protein. Supporting this idea are our results obtained from the analysis of single-channel kinetics of the H175K and K185E mutants. The H175K-mutant channel becomes completely insensitive to pH, owing to the lack of inhibition of the closed-time events, whereas its sensitivity to ATP is normal (Xu et al., 2001a). The K185E mutant, on the other hand, shows a typical suppression of the long and intermediate closures at acidic pH, as seen in the Kir6.2ΔC36. These results suggest that channel gating by these K_{ATP} channel regulators relies on distinct sites in the channel protein, although their sensitivities are modulated by each other. It is likely that the two binding sites can interact with each other when they are occupied, perhaps through an allosteric mechanism.

Our results indicate that protons affect the K_{ATP} by reducing the longer periods of closed states without evident changes in the open states. As a result, the mean closed time decreases and the P_{open} increases with moderate acidosis (Fig. 8*B*). In contrast, ATP expands the mean closed time with a moderate shortening of the mean open time (Drain, Li & Wang, 1998; Trapp et al., 1998; Babenko, Gonzalez & Bryan 1999; Enkvetchakul et al., 2000; Markworth, Schwanstecher & Schwanstecher, 2000) (Fig. 8*B*). With severe intracellular acidosis, the channels undergo rundown. Apparently, the major effects of these two K_{ATP} regulators are opposite to each other. Since they do not act on the same binding site in the channel protein, it is possible that the two K_{ATP} regulators share a common gating mechanism. Based on this hypothesis, there may be other protein domains and residues that are critical for both of these K_{ATP} regulators, including some that have been previously believed to be potential ATP-binding sites. Thus, the finding of the integrative modulation of the K_{ATP} by protons and ATP may help to understand the gating process of the K_{ATP} channels.

In conclusion, the present studies on single-channel kinetics show that the activation of the Kir6.2ΔC36 channel is mediated by selective inhibition of the long and intermediate closures, which is seen with and without ATP. The pH sensitivity is modulated by ATP although ATP and protons seem to bind to distinct sites in the channel protein. The pH sensitivity indicates that the K_{ATP} activity under a given cellular condition should be determined by all K_{ATP} regulators, including protons.

Special thanks to Dr. S. Seino for the gift of Kir6.2 cDNA. This work was supported by the N1H (HL58410), the American Diabetes Association (01039). CJ is a Career Investigator of the American Lung Association.

References

Allard, B., Lazdunski, M., Rougier, O. 1995. Activation of ATP-dependent K^+ channels by metabolic poisoning in adult mouse skeletal muscle: role of intracellular Mg^{++} and pH. *J. Physiol.* **485:**283–296

Ashcroft, F.M., Gribble, F.M. 1998. Correlating structure and function in ATP-sensitive K^+ channels. *Trends Neurosci.* **21:**288–294

Babenko, A.P., Gonzalez, G., Bryan, J. 1999. Two regions of sulfonylurea receptor specify the spontaneous bursting and ATP inhibition of K_{ATP} channel isoforms. *J. Biol. Chem.* **274:**11587–11592

Barry, P.H., Lynch, J.W. 1991. Liquid junction potentials and small cell effects in patch-clamp analysis. *J. Membrane Biol.* **121:**101–117

Baukrowitz, T., Schulte, U., Oliver, D., Herlitze, S., Krauter, T., Tucker, S.J., Ruppersberg, J.P., Fakler, B. 1998. PIP2 and PIP as determinants for ATP inhibition of K_{ATP} channels. *Science* **282:**1141–1144

Baukrowitz, T., Tucker, S.J., Schulte, U., Benndorf, K., Ruppersberg, J.P., Fakler, B. 1999. Inward rectification in K_{ATP} channels: a pH switch in the pore. *EMBO J.* **18:**847–853

Cuevas, J., Bassett, A.L., Cameron, J.S., Furukawa, T., Myerburg, R.J., Kimura, S. 1991. Effect of H^+ on ATP-regulated K^+ channels in feline ventricular myocytes. *Am. J. Physiol.* **261:**H755–H761

Davies, N.W. 1990. Modulation of ATP-sensitive K^+ channels in skeletal muscle by intracellular protons. *Nature* **343:**375–377

Davies, N.W., Standen, N.B., Stanfield, P.R. 1992. The effect of intracellular pH on ATP-dependent potassium channels of frog skeletal muscle. *J. Physiol.* **445:**549–568

Drain, P., Li, L., Wang, J. 1998. K_{ATP} channel inhibition by ATP requires distinct functional domains of the cytoplasmic C terminus of the pore-forming subunit. *Proc. Natl. Acad. Sci. USA* **95:**13953–13958

Enkvetchakul, D., Loussouarn, G., Makhina, E., Shyng, S.L., Nichols, C.G. 2000. The kinetic and physical basis of K_{ATP} channel gating: toward a unified molecular understanding. *Biophys. J.* **78:**2334–2348

Fan, Z., Makielski, J.C. 1997. Anionic phospholipids activate ATP-sensitive potassium channels. *J. Biol. Chem.* **272:**5388–5395

Fan, Z., Tokuyama, Y., Makielski, J.C. 1994. Modulation of ATP-sensitive K^+ channels by internal acidification in insulin-secreting cells. *Am. J. Physiol.* **267:**C1036–C1044

Findlay, I. 1992. Effects of pH upon the inhibition by sulphonylurea drugs of ATP-sensitive K^+ channels in cardiac muscle. *J. Pharmacol. Exp. Ther.* **262:**71–79

Gribble, F.M., Ashfield, R., Ammala, C., Ashcroft, F.M. 1997. Properties of cloned ATP-sensitive K^+ currents expressed in *Xenopus* oocytes. *J. Physiol.* **498** (Pt 1)**:**87–98

Inagaki, N., Gonoi, T., Clement, J.P., Namba, N., Inazawa, J., Gonzalez, G., Aguilar-Bryan, L., Seino, S., Bryan, J. 1995. Reconstitution of IK_{ATP}: an inward rectifier subunit plus the sulfonylurea receptor. Science **270:**1166–1170

Koyano, T., Kakei, M., Nakashima, H., Yoshinaga, M., Matsuoka, T., Tanaka, H. 1993. ATP-regulated K^+ channels are modulated by intracellular H^+ in guinea-pig ventricular cells. *J. Physiol.* **463:**747–766

Larsson, O., Ammala, C., Bokvist, K., Fredholm, B., Rorsman, P. 1993. Stimulation of the K_{ATP} channel by ADP and diazoxide requires nucleotide hydrolysis in mouse pancreatic beta-cells. *J. Physiol.* **463:**349–365

Lederer, W.J., Nichols, C.G. 1989. Nucleotide modulation of the activity of rat heart ATP-sensitive K^+ channels in isolated membrane patches. *J. Physiol.* **419:**193–211

Markworth, E., Schwanstecher, C., Schwanstecher, M. 2000. ATP4 mediates closure of pancreatic beta-cell ATP-sensitive potassium channels by interaction with 1 of 4 identical sites. *Diabetes* **49:**1413–1418

Mauerer, U.R., Boulpaep, E.L., Segal, A.S. 1998. Regulation of an inwardly rectifying ATP-sensitive K^+ channel in the basolateral membrane of renal proximal tubule. *J. Gen. Physiol.* **111:**161–180

Misler, S., Gillis, K., Tabcharani, J. 1989. Modulation of gating of a metabolically regulated, ATP-dependent K^+ channel by intracellular pH in B cells of the pancreatic islet. *J. Membrane Biol.* **109:**135–143

Noma, A. 1983. ATP-regulated K^+ channels in cardiac muscle. Nature **305:**147–148

Piao, H., Cui, N., Xu, H., Mao, J., Rojas, A., Wang, R., Abdulkadir, L., Li, L., Wu, J., Jiang, C. 2001. Requirement of multiple protein domains and residues for gating K_{ATP} channels by intracellular pH. *J. Biol. Chem.* **276:**36673–36680

Proks, P., Takano, M., Ashcroft, F.M. 1994. Effects of intracellular pH on ATP-sensitive K^+ channels in mouse pancreatic beta-cells. *J. Physiol.* **475:**33–44

Qu, Z., Yang, Z., Cui, N., Zhu, G., Liu, C., Xu, H., Chanchevalap, S., Shen, W., Wu, J., Li, Y., Jiang, C. 2000. Gating of inward rectifier K^+ channels by proton-mediated interactions of N- and C-terminal domains. *J. Biol. Chem.* **275:**31573–31580

Quayle, J.M., Nelson, M.T., Standen, N.B. 1997. ATP-sensitive and inwardly rectifying potassium channels in smooth muscle. *Physiol. Rev.* **77:**1165–1232

Reimann, F., Ryder, T.J., Tucker, S.J., Ashcroft, F.M. 1999. The role of lysine 185 in the Kir6.2 subunit of the ATP-sensitive channel in channel inhibition by ATP. *J. Physiol.* **520:**661–669

Repunte, V.P., Nakamura, H., Fujita, A., Horio, Y., Findlay, I., Pott, L., Kurachi, Y. 1999. Extracellular links in Kir subunits control the unitary conductance of SUR/Kir6.0 ion channels. *EMBO J.* **18:**3317–3324

Seino, S., Iwanaga, T., Nagashima, K., Miki, T. 2000. Diverse roles of K_{ATP} channels learned from Kir6.2 genetically engineered mice. *Diabetes* **49:**311–318

Shyng, S.L., Nichols, C.G. 1998. Membrane phospholipid control of nucleotide sensitivity of K_{ATP} channels. *Science* **282:**1138–1141

Trapp, S., Proks, P., Tucker, S.J., Ashcroft, F.M. 1998. Molecular analysis of ATP-sensitive K channel gating and implications for channel inhibition by ATP. *J. Gen. Physiol.* **112:**333–349

Tucker, S.J., Gribble, F.M., Zhao, C., Trapp, S., Ashcroft, F.M. 1997. Truncation of Kir6.2 produces ATP-sensitive K^+ channels in the absence of the sulphonylurea receptor. Nature **387:**179–183

Vivaudou, M., Forestier, C. 1995. Modification by protons of frog skeletal muscle K_{ATP} channels**:**effects on ion conduction and nucleotide inhibition. *J. Physiol.* **486:**629–645

Xu, H., Cui, N., Yang, Z., Wu, J., Giwa, L.R., Abdulkadir, L., Sharma, P., Jiang, C. 2001a. Direct activation of cloned K_{ATP} channels by intracellular acidosis. *J. Biol. Chem.* **276:**12898–12902

Xu, H., Wu, J., Cui, N., Abdulkadir, L., Wang, R., Mao, J., Giwa, L.R., Chanchevalap, S., Jiang, C. 2001b. Distinct histidine residues control the acid-induced activation and inhibition of the cloned K_{ATP} channel. *J. Biol. Chem.* **276:**38690–38696

Yang, Z., Xu, H., Cui, N., Qu, Z., Chanchevalap, S., Shen, W., Jiang, C. 2000. Biophysical and molecular mechanisms underlying the modulation of heteromeric Kir4.1-Kir5.1 channels by CO_2 and pH. *J. Gen. Physiol.* **116:**33–45

Zhu, G., Chanchevalap, S., Cui, N., Jiang, C. 1999. Effects of intra- and extracellular acidifications on single channel Kir2.3 currents. *J. Physiol.* **516:**699–710