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A Threonine Residue (Thr71) at the Intracellular End of the M1 Helix Plays a Critical Role in the Gating of Kir6.2 Channels by Intracellular ATP and Protons

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Abstract. ATP-sensitive K^+ (K_{ATP}) channels are known to be gated by several intracellular molecules, but the gating mechanisms remain unclear. To understand the relationship of channel gating to ligand binding, we studied Kir6.2 channel gating by ATP and protons, which inhibit and activate the channel, respectively. We have previously shown that a threonine residue (Thr71) is critical for the pH sensitivity of Kir6.2 channel. If this site is involved in channel gating rather than ligand binding, it should affect channel gating by both ATP and proton. To test this hypothesis we performed a mutation analysis. Site-specific mutations of Thr71 to a bulky residue reduced the ATP sensitivity by >100-fold and eliminated the pH sensitivity. Single-channel activity of these mutants was stabilized at the open state with no detectable rundown. Mutations to a small amino acid had little effect on the ATP and pH sensitivities. Mutations to intermediate amino acids reduced but did not abolish the ATP and pH sensitivities. Hydrophobicity is not critical, as both polar and nonpolar amino acids are found in each group. Mutation to a positively charged lysine markedly exacerbated the pH- but not ATP-sensitivity, whereas mutation to glutamate moderately reduced ATP and pH sensitivities. These results indicate that the residue mass is critical for Kir6.2 channel gating, a mass that should be below 120 daltons with no charge. The existence of such a site as Thr71 involved in channel gating by both ATP and proton suggests that channel gating in the K_{ATP} channel likely is separate from ligand binding.

Key words: K_{ATP} — Kir6.2 — Hypercapnia — ATP — pH — Diabetes — Coronary heart disease

Introduction

ATP-sensitive K^+ (K_{ATP}) channels are unique members in the ion-channel superfamily. Gated by intracellular ATP, these channels couple intermediary metabolism directly to cellular activity (Quayle et al., 1997; Ashcroft & Gribble, 1998; Yokoshiki et al., 1998). Such a property depends on the inherent ATP-sensing mechanisms that are known to be located in the Kir6 subunit. The ATP sensitivity has been widely studied over the past 6 to 7 years, with a large number of amino-acid residues involved having been revealed in several protein domains of the channel protein (Drain et al., 1998; Trapp et al., 1998; Tucker et al., 1998; Koster et al., 1999; Proks et al., 1999; Reimann et al., 1999). However, the fundamental question as to whether the ATP-binding site is a part of the channel gating mechanism or separate from the channel gating remains open. One way to address this question is to study these channels using two different K_{ATP} channel regulators that gate the channels via distinct ligand-binding sites.

The K_{ATP} channels are regulated by phospholipids, nucleotide diphosphates and hydrogen ion in addition to ATP (Davies, 1990; Koyano et al., 1993; Fan et al., 1994; Vivaudou & Forestier, 1995; Baukrowitz et al., 1998; Tucker et al., 1998; Shyng & Nichols, 1998; Xu et al., 2001a). As an important K_{ATP} channel activator, the proton has several advantages in the study of channel gating over PIP2 and ADP. Protons are less sensitive to their environment, and the manipulation of intra- and extracellular pH is rather easy. More importantly, we have recently shown that the proton-sensing mechanism in Kir6.2 is separate from the ATP sensing, and identified Hisl75 to be the proton sensor (Xu et al., 2001a,b). Our further studies have indicated that Kir6.2 gating by intracellular pH requires three protein domains, i.e., the N terminus, C terminus and M2 region (Piao et al., 2001).

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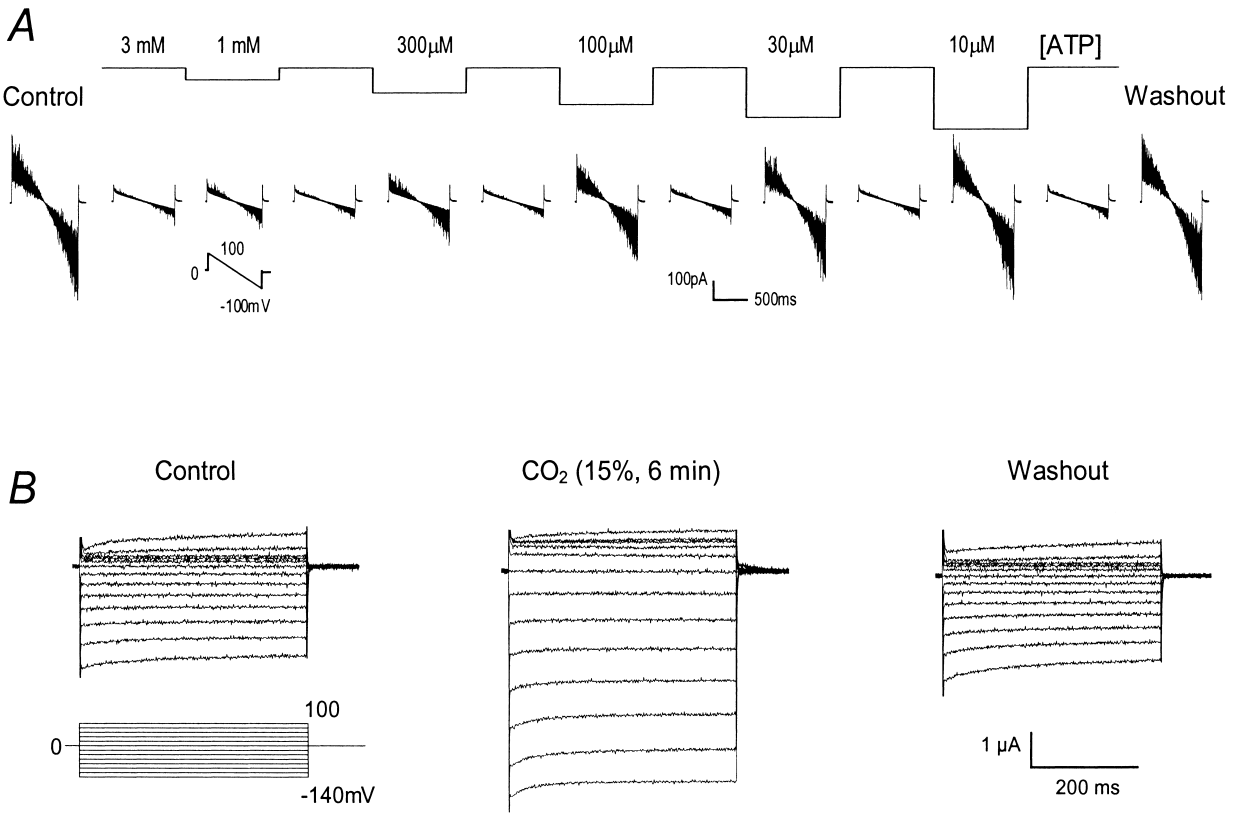


Fig. 1. Effects of ATP and protons on Kir6.2ΔC36 channels. (*A*) Kir6.2ΔC36 currents were recorded from an inside-out patch with equal concentrations of K^+ (145 mM) applied to both sides of the membrane. Command potentials from −100 mV to 100 mV were given at a holding potential of 0 mV. Under this condition, weak inward rectifying currents were recorded. These currents were inhibited by ATP (K^+ salt) with the mid-point inhibition of ~100 μM. Note that eight superimposed traces are shown in each panel, and there was no ATP in control and washout. (*B*) In whole-cell configuration, the Kir6.2ΔC36 currents were stimulated when the oocyte was exposed to 15% CO_2. This effect is also reversible. Currents returned to the baseline level 15 min after washout.

Threonine residue Thr71 is a critical player for the pH sensitivity in the N terminus (Piao et al., 2001). This residue is located at the cytosolic end of the M1 helix, or the PreM1 region that has been shown to be involved in pH sensitivity of several inward rectifier K^+ (Kir) channels (Fakler et al., 1996; Choe et al., 1997; McNicholas et al., 1998; Yang et al., 2000; Pessia et al., 2001). This as well as the fact that threonine cannot be titrated leads us to believe that this residue may play a role in channel gating rather than proton binding. If this residue is indeed involved in channel gating rather than maintaining the titratability of another protonation site, its mutations should affect channel gating by not only protons but also ATP. Demonstration of such a site that controls the channel sensitivities to both ATP and pH may provide information about the relationship of channel gating to ligand binding. Therefore, we performed a systematic mutation analysis on the Thr71, and studied channel sensitivities to both ATP and pH. To simplify the experimental subject, we chose to avoid the SUR subunit and used the Kir6.2 with 36 amino acids truncated in the C terminus, which expresses functional channels without the SUR (Tucker et al., 1997). Our results show that the Thr71 is involved in channel gating by both ATP and protons, suggesting that the channel-gating mechanism is separate from ligand binding in the Kir6.2 channel.

Materials and Methods

Oocytes

Oocytes from *Xenopus laevis* were used in the present studies (Piao et al., 2001; Xu et al., 2001a,b). 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). Then, 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 Chemicals) in the OR2 solution (in mM): NaCl 82, KCl 2, $MgCl_2$ 1 and HEPES 5, pH 7.4) for 90 min at room temperature. After 3 washes (10 min each) of the oocytes with the OR2 solution, cDNAs (25 to 50 ng in 50 nL water) were injected into the oocytes. The T71M mutant was studied, using cRNA (10 ng in 50 nL water). The oocytes were then incubated at 18 °C in the ND-96 solution containing (in mM) NaCl 96, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5, and sodium pyruvate 2.5 with 100 mg/L geneticin added (pH 7.4).

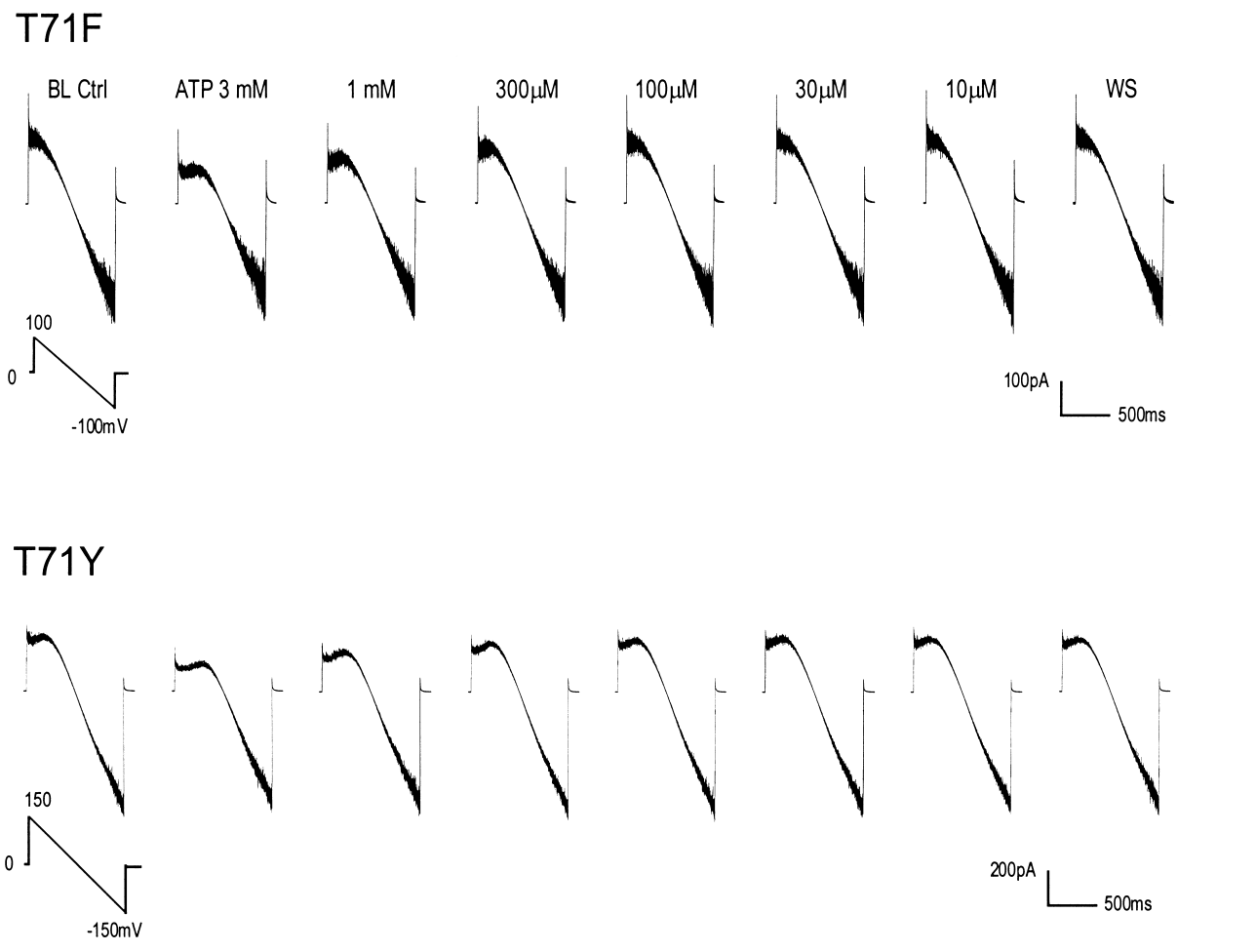


Fig. 2. The ATP sensitivity is greatly reduced with mutations of Thr71 to phenylalanine (T71F) or tyrosine (T71Y). The inward rectifying currents did not respond to ATP up to 1 mM, and were barely inhibited in 3 mM. The outward currents started being inhibited by 300 μM ATP with 50% inhibition in 1 to 3 mM. Note that eight superimposed traces are shown in each panel. Abbreviations: BL Ctrl, baseline control; WS, washout.

Mutant Construction

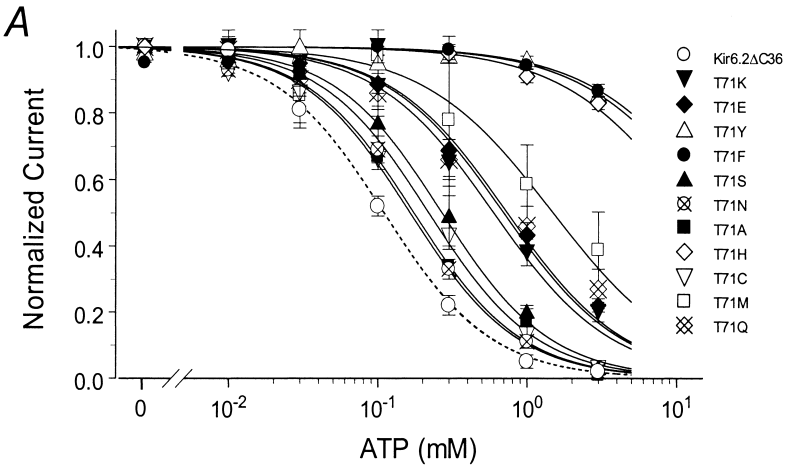
Mouse Kir6.2 (mBIR, GenBank # D50581) cDNA was generously provided by Dr. S. Seino and subcloned into a eukaryotic expression vector (pcDNA3.1, Invitrogen, Carlsbad, CA). The last 36 amino acids were deleted at the C terminal end (Kir6.2ΔC36,); this has previously been shown to render expression of the channel without the SUR subunit but with ATP sensitivity (Tucker et al., 1997). Approximately 1 μg purified linear DNA was used for cRNA synthesis using the Riboprobe In-vitro Transcription kit (Promega, Madison, WI) with the T7 promoter. After the DNA template was removed with DNase I, the RNA was precipitated with 4 M LiCl and 100% ethanol, washed twice with 70% ethanol, air-dried and resuspended in double-distilled water. Site-specific mutations were produced using a site-directed mutagenesis kit (Stratagene, La Jolla, CA). The correct mutations were confirmed with DNA sequencing (Piao et al., 2001; Xu et al., 2001 a,b). The potential changes in the peptide backbone in these mutations were examined using the PHDsec and Chou-Pasman analyses. Only mutations that did not have a major effect on the secondary structures were used in further studies.

Whole-cell Currents

Whole-cell currents were studied on the oocytes 2 to 4 days after injection (Piao et al., 2001; Xu et al., 2001a,b). Two-electrode voltage clamp was performed using an amplifier (Geneclamp 500, Axon Instruments, Foster City, CA) at room temperature (~24°C). The extracellular solution contained (in mM): KCl 90, $MgCl_2$ 3, and HEPES 5 (pH 7.4).

Patch Clamp

Patch clamp was 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 (Yang et al., 2000). Pyrophosphate and vanadate are known to alleviate channel rundown. In several control experiments, we did not find any evident difference in current profile and channel responses to pH and ATP from those recorded in the absence of pyrophosphate and vanadate. Single-channel conductance was measured using ramp-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 (Yang et al., 2000). The P_{open} was then obtained as $P_{open} = (\Sigma_{j=1}^{N} t_j \cdot j)/TN$, where N is the number of channels active in the patch and T is the duration of recordings. P_{open} values were



B

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52                          M1·····
|                          ┌
QG-RFLQDVFTTLVDLKWPHTLL......Kir6.2
QG-RFLQDIFTTLVDLKWRHTLV......Kir6.1
SRFIFFVDIWTTVLDLKWRYKMT......Kir1.1
KGQRYLADIFTTCVDIRWRWMLV......Kir2.1
KSQRYMADIFTTCVDTRWRYMLM......Kir2.3
T-YRYLSDLFTTLVDLKWRFNLL......Kir3.4
KRFLYLKDLWTTFIDMQWRYKLL......Kir4.1
EWGSYMVDIFTTLVDTKWRHMFV......Kir5.1
LSGLLARLVKLLLGRHGSALHWR......KcsA
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Fig. 3. (*A*) Dose-dependent inhibition of the Kir6.2ΔC36 and its mutants. Currents were studied in the same condition as in Fig. 1*A*. The current-ATP relationship is expressed using the Hill equation $y = 1/(1 + ([ATP]/IC50)^h)$, where y = normalized currents, [ATP] = ATP concentration, IC50 = the mid-point channel inhibition, and h = the Hill coefficient. The IC50 for each channel is shown in Table 1, and the h values are 1.0 to 1.2. (*B*) Alignment of about 20 amino acid residues in the region immediately before the M1 helix in several Kir channels. Residues found in the same location of Thr71 are printed bold.

calculated from one to three stretches of data acquired using the Fetchex software (Axon Instruments) and having a total duration of 20 to 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). Owing to the limitation of sampling speed (50 μs), open/closed events ≤100 μs cannot be surely calculated. Therefore, events <100 μs were ignored.

A parallel perfusion system was used to deliver perfusates with different concentrations of ATP (K^+ Salt) and proton at a rate of ~1 mL/min with no dead space (Yang et al., 2000; Xu et al., 2001a). The pH levels were adjusted after ATP was completely dissolved. All solutions with ATP were prepared immediately before experiments and were used for less than 4 hours.

Experiments were performed in a semi-closed recording chamber (BSC-HT, Medical System, Greenvale, NY), 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. Low pH was produced by superfusing the cells with a gas mixture of 15% CO_2 balanced with 21% O_2 and N_2. The high solubility of CO_2 resulted in a detectable change in intra- or extracellular acidification as fast as 10 s in these oocytes. The corresponding changes in intra- and extracellular pH levels with the CO_2 exposure have been well characterized in our previous studies, which are pH 6.6 and 6.2, respectively (Paio et al., 2001. Xu et al., 2001a,b). Since the extracellular pH has no effect on the Kir6.2 channel, only the intracellular pH was considered in the present study.

Data Analysis

Channel sensitivities to ATP and protons were plotted against the residue mass of amino acid with which the Thr71 was replaced. The residue mass was obtained according to Voet & Voet (1990). Data are presented as means ± SE (standard error). ANOVA or Student's t-test was used. Differences of CO_2 and pH effects before versus during exposures were considered to be statistically significant if $P \leq 0.05$.

Results

Kir6.2ΔC36 channels were studied in *Xenopus* oocytes that had received a cDNA injection 2 to 4 days earlier. K^+ currents were recorded in the two-electrode voltage-clamp mode using a bath solution (KD90) containing 90 mM K^+. Currents showed clear inward rectification with an amplitude of −2.1 ± 0.3 μA (n = 14, measured at −160 mV). These currents were inhibited by Ba^{2+} in micromolar concentrations (*not shown*). Exposure to 3 mM azide (Gribble et al., 1997) strongly activated the Kir6.2ΔC36 currents (−7.6 ± 1.7 μA, n = 6). In contrast, oocytes that received an injection of the expression vector alone

Table 1. CO_2 and ATP sensitivities of wt and mutant Kir6.2 channels

Name	BL Current (μA)	% pH effect	BL P_{open}	IC50 ATP (mM)
Kir6.2ΔC36	2.1 ± 0.3 (14)	142.3 ± 12.1 (14)	0.029 ± 0.005 (7)	0.11 (8)
T71A	3.0 ± 0.3 (6)	153.2 ± 9.4 (6)	0.017 ± 0.004 (5)	0.17 (4)
T71P	NF	NF	NF	NF
T71N	4.8 ± 0.6 (5)	167.1 ± 19.3 (5)	0.034 ± 0.007 (4)	0.17 (4)
T71C	2.2 ± 0.3 (5)	155.6 ± 18.9 (5)	0.103 ± 0.012 (4)	0.22 (4)
T71S	4.5 ± 0.5 (4)	118.5 ± 9.6 (4)	0.139 ± 0.037 (4)	0.27 (4)
T71Q	1.9 ± 0.5 (8)	43.8 ± 9.0 (14)	0.117 ± 0.014 (7)	0.70 (11)
T71M	1.6 ± 0.2 (11)	59.2 ± 8.7 (11)	0.065 ± 0.009 (7)	1.50 (4)
T71H	13.0 ± 3.7 (4)	10.1 ± 5.1 (4)	0.762 ± 0.022 (6)	>11 (4)
T71F	36.2 ± 4.4 (4)	1.0 ± 1.1 (4)	0.825 ± 0.051 (4)	>15 (4)
T71Y	35.3 ± 4.1 (6)	0.7 ± 1.6 (6)	0.770 ± 0.040 (6)	>16 (4)
T71K	2.4 ± 0.3 (12)	−13.9 ± 4.2 (12)	0.171 ± 0.033 (4)	0.60 (5)
T71R	NF	NF	NF	NF
T71E	22.0 ± 3.1 (11)	44.0 ± 11.1 (11)	0.452 ± 0.103 (4)	0.75 (5)
T71D	NF	NF	NF	NF

The pH effect was studied by exposing the oocytes to 15% CO_2 for 5 to 6 min. The ATP sensitivity is expressed as IC50 levels obtained by fitting data using the Hill equation shown in Fig. 3*A*. In T71H, T71F, T71Y and T71K, the regression curves were not fully supported by data points. Therefore, we deliberately reduced the IC50 values shown in this Table by 20% from those used for data-fitting in Fig. 3*A*. Abbreviations: BL current, whole-cell baseline current; BL P_{open}, baseline open state probability; NF, nonfunctional. Data are shown as means ± SE with *n* in parenthesis.

did not express such inward-rectifying currents. The small currents displayed (−0.1 to 0.4 μA) were insensitive to Ba^{2+} and azide.

Channel activity was studied in inside-out patches after the expression of inward rectifying currents was identified in each cell. These patches were exposed to symmetric concentrations of K^+ (145 mM) on both sides of the plasma membrane with command potentials from −100 mV to 100 mV applied through the recording pipette. Under such conditions, the inward Kir6.2ΔC36 currents showed a single-channel conductance of ~70 pS and were inhibited by ATP (K^+ salt) in a concentration-dependent fashion. The ATP concentration for 50% channel inhibition was ~100 μM (Fig. 1*A*).

In the whole-cell configuration, the Kir6.2ΔC36 channel was strongly activated during an exposure to 15% CO_2. The channel activation was fast and fully reversible (Fig. 1*B*). At the end of a 6 min exposure, CO_2 enhanced the current amplitude by ~130%, which is consistent with our previous observation (Piao et al., 2001; Xu et al., 2001a,b). To avoid channel rundown seen at acidic pH in excised patches (Xu et al., 2001a,b), the pH experiments in the present study were mostly performed in whole-cell recordings using hypercapnic acidosis (produced by 15% CO_2).

We have previously shown that Thr71 is involved in the pH sensitivity, even though it is not a titratable residue (Piao et al., 2001). To elucidate whether mutations of this residue affect the channel gating, we examined the channel sensitivities to ATP and pH in these mutants. Site-specific mutations of the Thr71 to residues with a bulky side chain (nonpolar phenylalanine or neutral polar tyrosine) had drastic effects on the ATP and pH sensitivities. The T71Y and T71F mutant channels did not respond to 1 mM ATP and were only slightly inhibited by ATP in 3 mM (Fig. 2). The IC50 level of ATP inhibition was estimated to be greater than 15 mM (Fig. 3*A*). Remarkably, we found that the outward currents were still inhibited by high concentrations of ATP (Fig. 2). The IC50 for these outward currents was ~3 mM, suggesting that the gating mechanisms for inward and outward currents may be different. In whole-cell configuration, the T71Y and T71F mutant channels showed very large baseline currents with rather weak inward rectification (Fig. 4*A*). When they were exposed to 15% CO_2, no detectable changes in either inward or outward currents were found (Fig. 4*A*, Table 1). These results indicate that a bulky residue at the location of Thr71 interrupts the channel gating by both ATP and pH.

At the same position of Thr71, a hydrophobic residue methionine is found in Kir2.x, Kir5.1 and Kir7.1 channels (Fig. 3*B*). We thus mutated the Thr71 to methionine. The T71M mutation markedly reduced the ATP and pH sensitivities. However, neither of these effects was as great as that in the T71F and T71Y. The mutant channel was inhibited by 50% when the ATP concentration reached 1.5 mM (Fig. 3*A*), while 15% CO_2 stimulated the currents by ~60% (Fig. 4*B*, Table 1). Since the hydrophobicity of threonine is different from methionine, the decrease in ATP and pH sensitivities in the T71M mutation implies that hydrophobicity may be critical for the channel gating. We therefore performed studies by introducing another mutant (T71A). With hydrophobic alanine at this site, the mutant channel only showed a modest decrease in its ATP sensitivity (IC50 170 μM, Table 1; also Figs. 3*A* and 5), and its CO_2 sensitivity was almost the same as that of

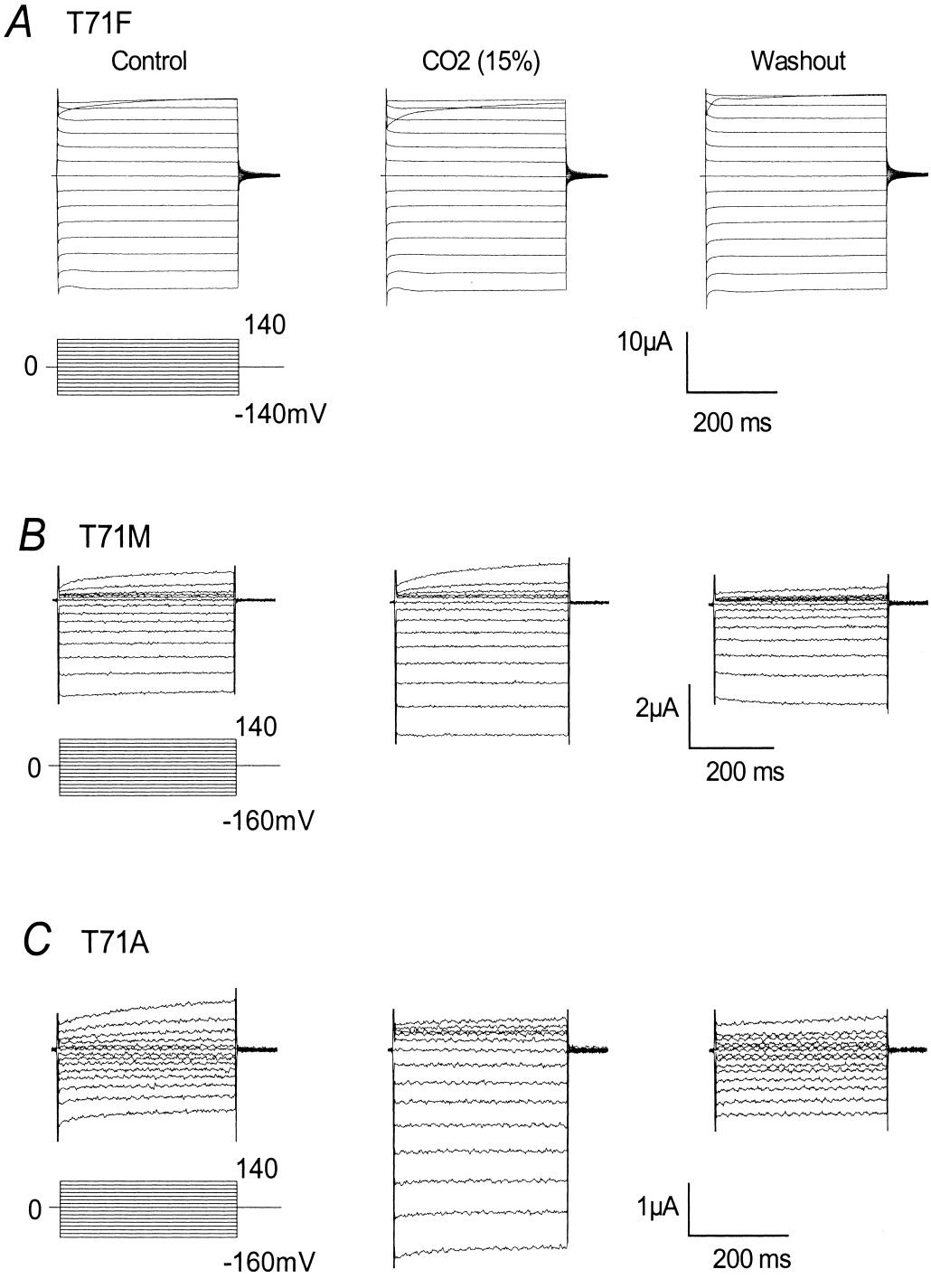

Fig. 4. The pH sensitivity of Thr71 mutant channels. Whole-cell currents were studied in oocytes receiving an injection of one of the Thr71 mutants. The pH sensitivity was studied by exposing the cells to 15% CO_2 for 6 min. (*A*) Large baseline currents were recorded from the T71F. These currents did not respond to a 6 min exposure to 15% CO_2. (*B*) The T71M mutation produced intermediate baseline currents. They were modestly stimulated by the same concentration of CO_2. (*C*) Although the baseline currents of T71A were small, they were strongly activated by 15% CO_2.

Kir6.2ΔC36 (Fig. 4*C*), suggesting that hydrophobicity is not critical. Subsequently, we mutated the Thr71 to other small residues, cysteine and serine. The T71C and T71S showed high pH sensitivity with a slight decrease in their ATP sensitivity (Table 1). Thus, residue mass rather than hydrophobicity at the Thr71 site seems important for the channel gating by ATP and pH.

To further understand the role of residue mass, we constructed several mutant channels with inter-

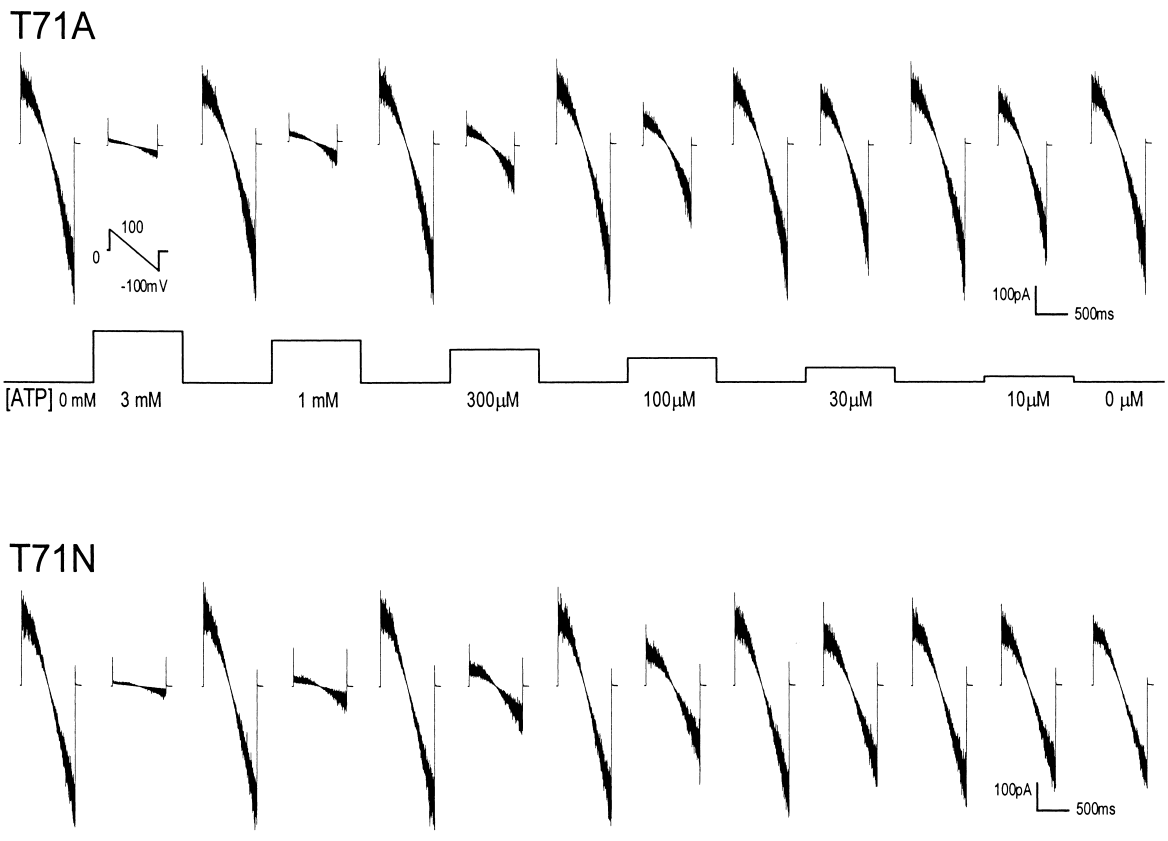

Fig. 5. ATP sensitivity of T71A and T71N. Currents were studied in the same condition as in Fig. 1*A*. Unlike the T71F, both T71A- and T71N-mutant channels remained highly sensitive to ATP. Note that eight superimposed traces are shown in each panel.

mediate residue mass. Similar to threonine, asparagine is a polar non-charged residue with a residue mass (114 daltons) slightly smaller than methionine (131 daltons), and is seen at the same location in Kir3x channels (Fig. 3*B*). The T71N mutant expressed functional channels like its wild-type counterpart, with clear inward rectification. The ATP sensitivity of the T71N mutant was very close to its wild-type counterpart (IC50 170 μM) (Figs. 3*A* and 5, Table 1), and it responded to hypercapnic acidosis almost identically to the Kir6.2ΔC36 channel (Table 1). Glutamine is another polar non-charged amino acid with a residue mass (128 daltons) in between methionine and asparagine. When the T71Q mutant was examined, we found that its pH sensitivity had dropped by 50% and the IC50 concentration of ATP increased by 7-fold. Histidine is a highly hydrophilic alkaline residue with a residue mass of 137 daltons. With a histidine at this location, both ATP- and pH-sensitivities were greatly diminished (Table 1). The channel activation during CO_2 exposure was almost abolished, and its ATP sensitivity became >100 times lower than the Kir6.2ΔC36.

When channel responses to ATP and pH were plotted as a function of the residue mass (Fig. 6), we surprisingly found that the effects of ATP and pH on channel activity were almost identical. All mutant channels with a residue smaller than asparagine were strongly activated by protons and inhibited by ATP, while channels with a residue larger than histidine were barely affected by ATP and pH (Table 1). The transition occurs between 120 and 140 daltons (Fig. 6). Consistently, the baseline P_{open} also varied with the residue mass (Table 1). The transition from low P_{open} to high P_{open} also takes place at the residue mass of 120 to 140 daltons (Fig. 6). These results therefore strongly suggest that the residue mass is the determinant for Kir6.2 channel gating by ATP and pH at the Thr71 site.

To ascertain how the charged status affects the ATP/pH sensitivities, the Thr71 was also mutated to residues with positive or negative charge at pH 7.4. We have previously shown that the T71K mutation eliminates the pH sensitivity in Kir6.2 channel (Piao et al., 2001). Indeed, the T71K mutant was slightly inhibited, rather than activated, by hypercapnic acidosis (Table 1). We found that the T71K-mutant channel had very low ATP sensitivity. The channel was only modestly inhibited by 300 μM ATP with an IC50 concentration of ATP of 0.6 mM (Figs. 3*A* and 7, Table 1). The low ATP sensitivity did not produce large whole-cell currents or high baseline P_{open} (Table 1), making the T71K distinct from T71Y and T71F. When the Thr71 was replaced with glutamate, an

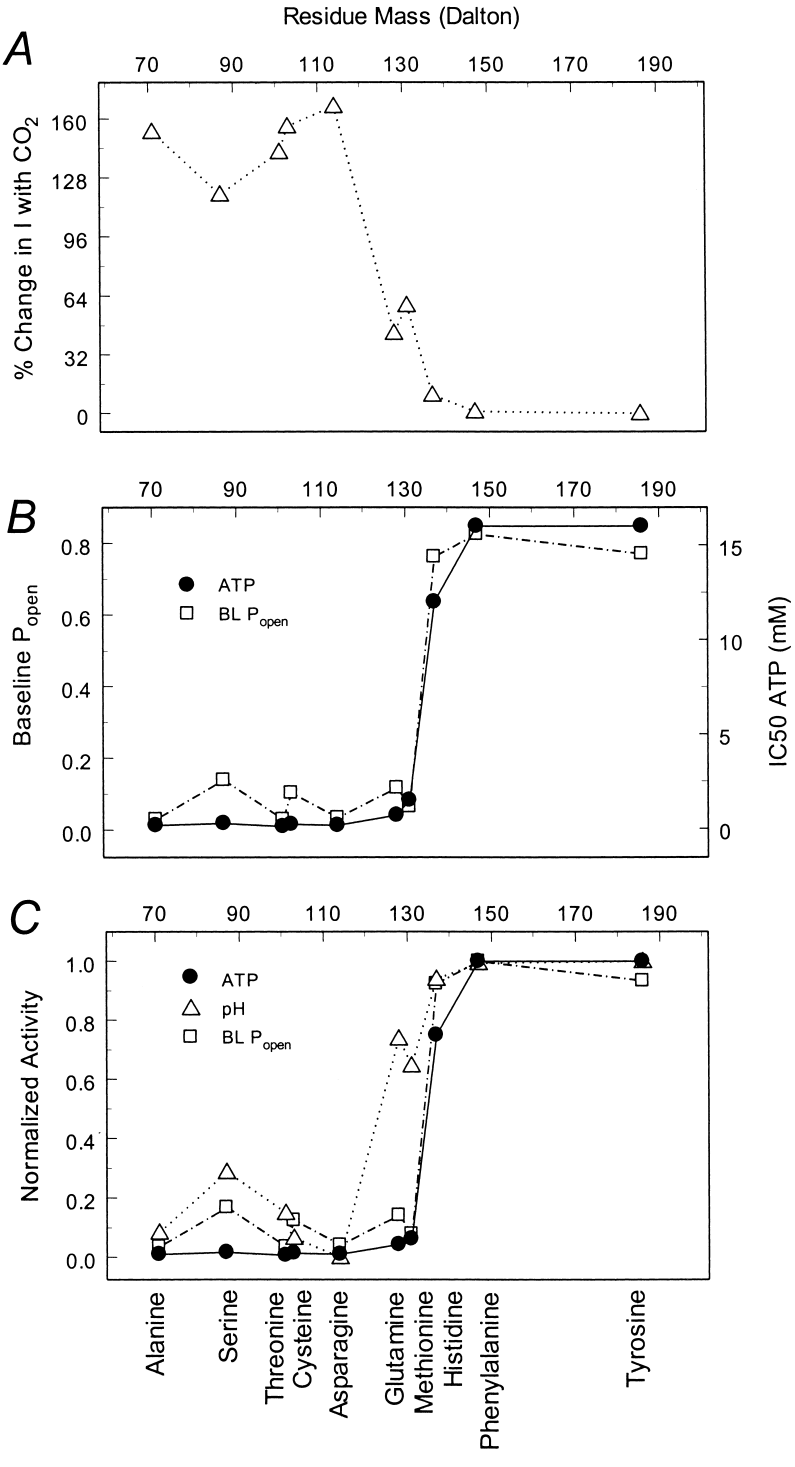

Fig. 6. Residue mass dependence. (*A*) The effect of CO_2 on whole-cell currents was plotted against the residue mass at the Thr71 location. A marked reduction in the pH sensitivity occurred with residues larger than methionine (131 daltons). (*B*) Similar effects were seen in the ATP sensitivity (IC50 ATP) and baseline channel activity (BL P_{open}). The channels showed high ATP sensitivity and small baseline P_{open} with residue mass smaller than 131 daltons. In contrast, low ATP sensitivity and large baseline P_{open} were found with residue mass greater than 137 daltons. (*C*) When all plots in *A* and *B* are pooled together, the critical mass of this residue is shown to be 120 to 140 daltons for the ATP sensitivity, pH sensitivity and baseline channel activity. Note that the plot in *A* (pH) was inverted for the comparison purpose.

acidic amino acid, both the ATP- and pH-sensitivities were significantly reduced (Figs. 3*A* and 7). In contrast to the T71K, the T71E showed high baseline P_{open} and large whole-cell currents (Table 1). Mutations to aspartic acid and arginine did not produce functional channels.

Consistent with our previous studies on macroscopic currents, single-channel Kir6.2ΔC36 currents showed a biphasic response to acidic pH in inside-out patches. Channel activity increased with moderate acidification and was strongly inhibited at extremely acidic pH (Fig. 8). Whereas a similar response was observed in the T71A mutant, the T71Y and T71H became completely insensitive to the pH change from 7.4 to 6.2 (Fig. 8), and did not show rundown. The T71M showed intermediate activation at acidic pH,

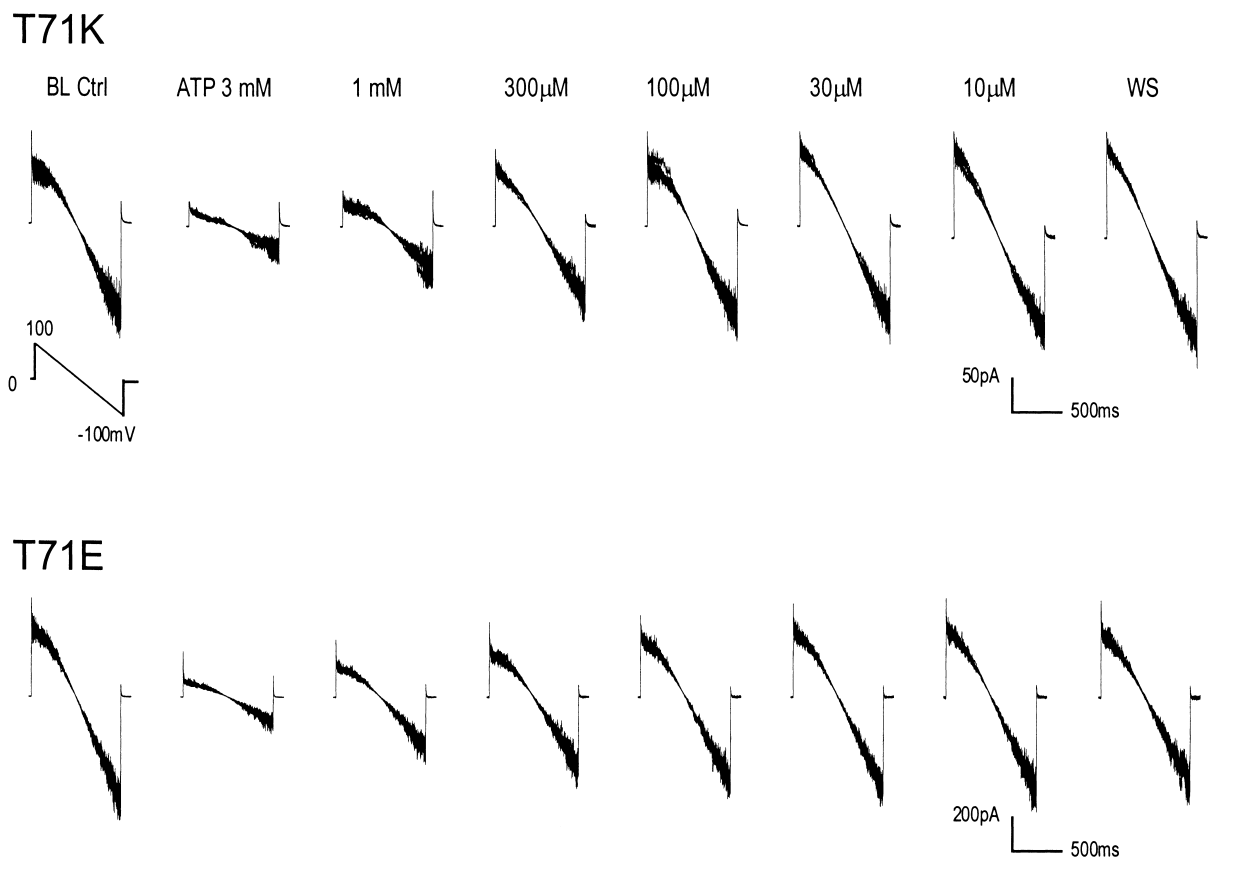

Fig. 7. Dose-dependent inhibition of the T71K and T71E mutant channels by ATP. Currents were studied in the same condition as in Fig. 1*A*. The ATP sensitivity of T71K was low. This mutant was modestly inhibited by ATP at 3 mM. The T71E started being inhibited by ATP at 300 μM. Marked inhibitions were seen with ATP in 1 and 3 mM. Note that these channels did not show marked rundown. Abbreviations: BL Ctrl, baseline control; WS, washout.

whereas the T71K was inhibited. The baseline channel activity before acid exposure was low in Kir6.2ΔC36 and T71A, and very high in T71Y and T71H mutants. The T71M and T71K showed intermediate baseline channel activity (Fig. 8).

Discussion

To our knowledge, this is the first demonstration of an amino-acid residue contributing to Kir channel gating by two different ligands. This contribution depends on residue mass rather than other side-chain properties of the amino acid, with the critical mass between 120 and 140 daltons.

The Thr71 is located at the border between the N terminus and the M1 helix. Based on the structural information of bacterial KcsA channels, this residue faces the M2 region and may be involved in the interaction of the M1 with the M2 helix (Doyle et al., 1998; Lu, Zhu & Yang, 1999; Capener et al., 2000; Minor et al., 2000). Because of its special location, this site is known to play a crucial role in channel gating by intracellular protons in a number of Kir members such as Kirl.l, Kir1.2, Kir4.1 and Kir4.2 (Fakler et al., 1996; Choe et al., 1997; McNicholas et al., 1998; Yang et al., 2000; Pessia et al., 2001) as well as Kir6.2, as shown in the present study. There is a lysine at this site in Kirl and Kir4 channels, mutation of which to a methionine completely eliminates the pH sensitivity in these channels (Fakler et al., 1996; Choe et al., 1997; McNicholas et al., 1998; Yang et al., 2000; Pessia et al., 2001). Although this lysine is believed to be protonated at acidic pH (Fakler et al., 1996; Schulte et al., 1999), several titratable histidine residues in the C terminal are also critical for the pH sensitivity of Kirl.l channels (Chanchevalap et al., 2000). Thus, whether this lysine residue is a proton sensor or a part of the channel gating mechanisms remains unclear. Unlike lysine, Thr71 in the Kir6.2 channel is non-titratable. Thereby, it may affect the pH sensitivity by either maintaining the titratability of another protonation site or by contributing to the channel gating. Using two Kir6.2 channel regulators (ATP and proton), we have differentiated these two possibilities. We have found that the Thr71 is required for not only channel activation by protons but also for channel inhibition

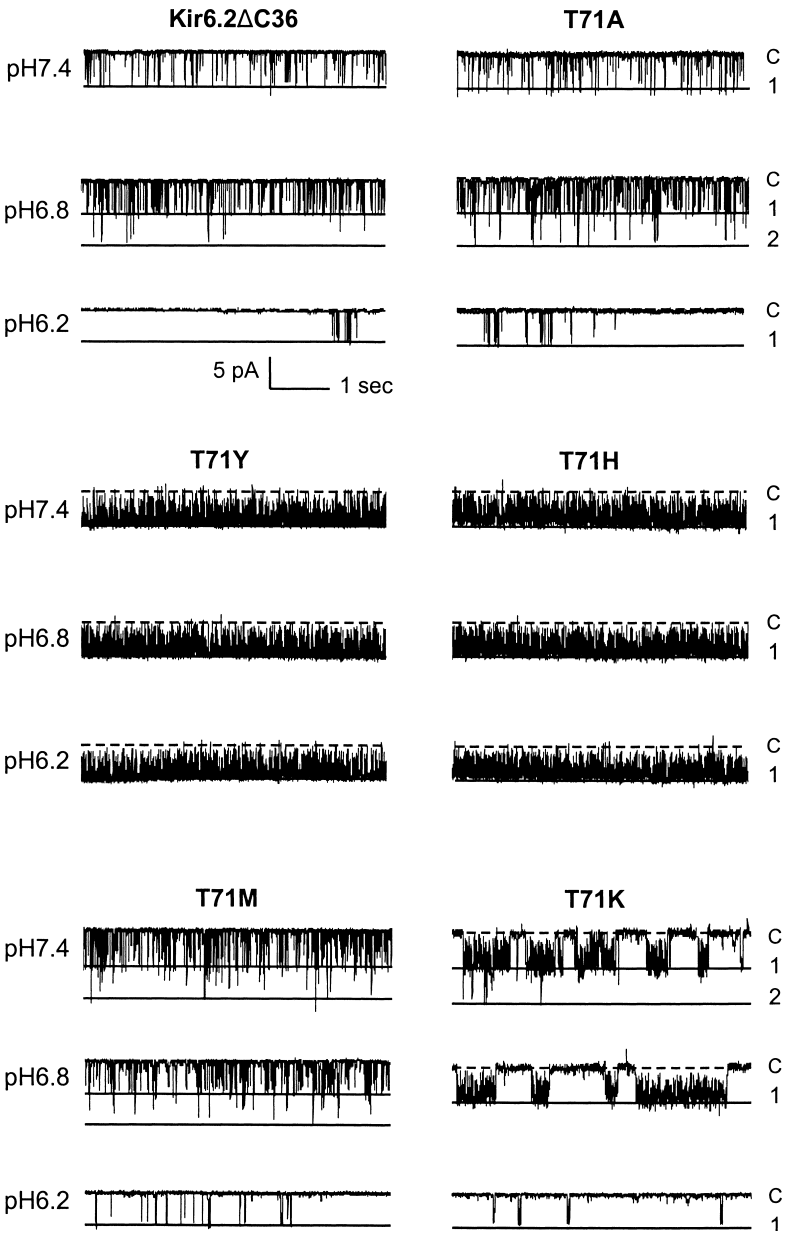

Fig. 8. Responses of single-channel currents to acidic pH. Currents were recorded from inside-out patches with a holding potential of −80 mV and exposed to a series of pH levels of the internal solution. The Kir6.2ΔC36 shows a biphasic response to acidic pH. Currents were stimulated at pH 6.8 and inhibited at pH 6.2. While a similar pattern was seen in the T71A currents, the T71Y and T71H did not respond to acidic pH. The T71M was moderately stimulated at pH 6.2, and the T71K was inhibited. Labels on the right indicate numbers of openings with *C* as closure, *1*, the first opening, and *2*, the second opening. The pH levels are shown on the left.

by ATP. Thus, the Thr71 should be responsible for sensing not just one of the K_{ATP} channel regulators. Since proton sensing in Kir6.2 has been shown to be separate from ATP sensing (Wu et al., 2002), our results suggest that the Thr71 is involved in channel gating rather than ligand binding. In addition, our results also suggest that the ligand-mediated activation and inhibition share the same gating mechanism in the Kir6.2 channel, which appears to be another novel finding from the present study.

Residue mass is the determinant for channel gating at the Thr71 site. Threonine can be phosphorylated. However, Thr71 phosphorylation is not necessary for the channel gating, because replacement of this residue with asparagine, cysteine or alanine has very little effect on the ATP and pH sensitivities. Hydrophobicity is not critical either, as the hydrophilic threonine can be replaced with a hydrophobic alanine without major compromises of the ATP and pH sensitivities. Similarly, the ATP and pH sensitivities are both interrupted by mutations to tyrosine or phenylalanine that are hydrophilic and hydrophobic, respectively. When the effects of ATP and pH on channel activity are plotted as a function of residue mass, the mass dependence becomes clear. The channels are well gated with small residues such as alanine, threonine, asparagine, etc. With a bulky residue at the Thr71 site, the mutant channels are stabilized at the open state and lose the ATP and pH sensitivities. This observation is consistent with a previous report showing that mutations of Asn94 to histidine or phenylalanine in Kir3.2 lead to consti-

tutively active channels, while mutations to other residues fail to rescue yeast that lack TRK1 and TRK2 and that display no detectable currents in *Xenopus* oocytes (Yi et al., 2001). Thus, the critical mass of this residue that we have estimated based on the plots in Fig. 6 is 120 to 140 daltons.

With a positively charged lysine at the Thr71 site, the channel sensitivity to ATP decreases, and the pH-dependent channel activation is abolished. The mutant channel was slightly inhibited at acidic pH, which is consistent with a brief comment by Schulte et al. (1999), although detailed studies have not been reported. When the Thr71 is mutated to a negatively charged glutamate, the channel shows intermediate sensitivities to ATP and pH, suggesting that the charge state also plays a role. The charge state at the Thr71 site also affects the baseline channel activity. The baseline P_{open} is 0.17 in the T71K and 0.45 in the T71E, leading to smaller whole-cell currents in the T71K than in the T71E. The residue mass of lysine is almost identical to that of glutamate (129 versus 128 daltons). Thereby, the residue mass should not cause the differences between these mutant channels, although it may contribute to the reductions in their ATP and pH sensitivities. It is possible that the side-chain shape has also an effect on the channel activity. Alignment of the amino-acid sequences of several Kir channels shows a conserved lysine residue (Lys170 in Kir6.2) at the inner mouth of the M2 helix. According to the KscA channel model (Doyle et al., 1998), the Lys170 is located in the immediate vicinity of the Thr71 (we have studied the Lys170 and found that its mutations did not yield any functional channels; unpublished observations by Wang, Rojas & Jiang). Thus, mutation of the Thr71 to a charged residue may create additional electrostatic interaction of the M2 helix with the M1 and limit their mobility. Consistent with this idea, the residue-mass requirement also suggests that protein domains around Thr71 and even the N terminus are movable during the channel gating process. A bulky residue and electrostatic forces between the M1 and M2 may confine the movement. It is also possible that a bulky residue and electrostatic forces hinder or even cause tilting of the M2 helical bundle, and thus prevent its conformational change necessary for channel gating. Thereby, the channels can be neither inhibited by ATP nor activated by protons.

We believe that the identification of a site serving for channel gating but not ligand binding has a major impact on the understanding of the K_{ATP} channel function and modulation. The existence of such a site indicates that channel gating is separate from ligand binding in the K_{ATP} channel. This information can help simplify experimental procedures, and formulate new approaches to each of the channel-gating and ligand-binding mechanisms. Since the gating mechanisms in the K_{ATP} channel may be shared by several other intracellular ligand-gated ion channels, our observations also contribute useful information to the understanding of their gating mechanisms.

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