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Determinant Role of Membrane Helices in K_{ATP} Channel Gating

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Abstract. The ATP-sensitive K^+ (K_{ATP}) channels couple chemical signals to cellular activity, in which the control of channel opening and closure (i.e., channel gating) is crucial. Transmembrane helices play an important role in channel gating. Here we report that the gating of Kir6.2, the core subunit of pancreatic and cardiac K_{ATP} channels, can be switched by manipulating the interaction between two residues located in transmembrane domains (TM) 1 and 2 of the channel protein. The Kir6.2 channel is gated by ATP and proton, which inhibit and activate the channel, respectively. The channel gating involves two residues, namely, Thr71 and Cys166, located at the interface of the TM1 and TM2. Creation of electrostatic attraction between these sites reverses the channel gating, which makes the ATP an activator and proton an inhibitor of the channel. Electrostatic repulsion with two acidic residues retains or even enhances the wild-type channel gating. A similar switch of the pH-dependent channel gating was observed in the Kir2.1 channel, which is normally pH-insensitive. Thus, the manner in which the TM1 and TM2 helices interact appears to determine whether the channels are open or closed following ligand binding.

Key words: Ion channel — Channel gating — ATP — pH.

Introduction

The inward rectifier K^+ channels are controlled by second messengers or metabolites and couple the chemical signals to cellular excitability (Nichols & Lopatin, 1997). To a given intracellular ligand, a channel species has a stereotypical response, i.e., stimulation or inhibition of channel activity, indicating that such a response is an inherent property of the channel protein. In contrast to the channel response, a given ligand molecule often has diverse effects on distinct channel species. For instance, ATP is an antagonist of the ATP-sensitive K^+ (K_{ATP}) channels, whereas it augments the Kir1.1 channel activity (Ho et al., 1993; Noma, 1983;). Although the Kir6.1 and Kir6.2 channels are stimulated by intracellular proton (Davies, 1990; Davies standen & Stanfield, 1992; Xu et al., 2001), the Kir1.1, Kir2.3, Kir4.1 and Kir4.1-Kir5.1 channels are strongly inhibited (Coulter et al., 1995; Tsai et al., 1995; Fakler et al., 1996; Yang & Jiang, 1999; Zhu et al., 1999; Yang et al., 2000). How these opposite responses are produced is unknown. The current understanding is that ligand binding produces a specific conformation of the ligand-binding domain, which is commensurate with the channel activity, suggesting that the channel response is determined by the ligand-binding domains. According to its conformation with or without ligand binding, the opening or closure of a channel takes place.

Another possibility is that the channel response to a ligand molecule may depend not only on the conformation of the ligand-binding domains but also on the whole assembly of the gating apparatus, which is likely to consist of the transmembrane helices and their adjacent regions of the N and C termini (Yellen, 2002). Acting as the body of the gating assembly, the TM2 helices are assembled as an inverted teepee lining the inner ion-conductive pore (Doyle et al., 1998). Experimental evidence suggests that the narrowest part of the TM2 helices is widened when the channels are open, allowing certain ions and organic molecules to access the inner cavity (Armstrong et al., 1966; Liu et al.,1997; del Camino et al., 2000; Enkvetchakul et al., 2001; Flynn & Zagotta, 2001; Shin, Rothberg & Yell, 2001; Jin et al.,

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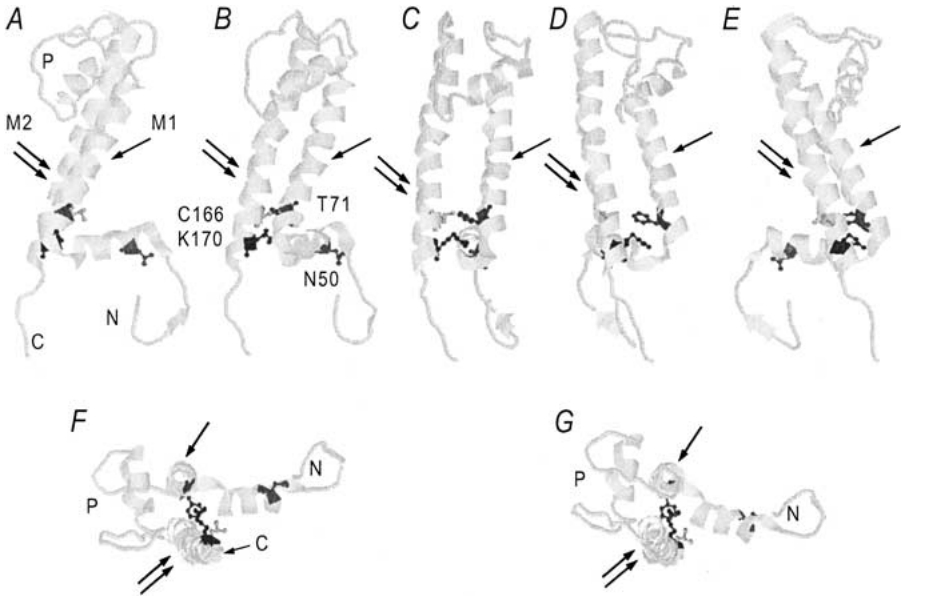

Fig. 1. Relative locations of Thr71, Cys166 and Lys170. Amino-acid sequence of Kir6.2 was aligned with that of KirBaC1.1 and a subunit with the first (*M1*, *single arrow*) and second (*M2*, *double arrow*) membrane helices displayed, based on the crystal structure of the KirBac1.1 (Kuo et al., 2003). Thr71 (T71), Cys166 (C166) and Lys170 (K170) in the Kir6.2 are shown in the locations of Phe63, Leu144 and Arg148 in the KirBac1.1, respectively. The distance measured using RasMol (Version 2.6) is 4.7 Å between Thr71 and Cys166, and 5.2 Å between Thr71 and Lys170. Also shown is Asp50 (N50), which has been suggested to interact with Arg148 with a distance of 17.1 Å (Kuo et al., 2003). (*A–E*) Side view of the M1 and M2 helices from one subunit with a 40° counter-clockwise rotation, as seen extracellularly in each panel. Two lower panels are intra- (*F*) and extracellular (*G*) views of the subunit. Other abbreviations: *C*, C-terminus; *N*, N-terminus; *P*, pore loop,

2002; Phillips, Enkvetchakul & Nichols, 2003). Such a movement could be produced solely by the ligand binding. If so, the gating assembly would be a simple follower of the ligand-binding domains, which are located in the intracellular termini. However, some studies suggest that the membrane helices may play a more active role in channel gating, as mutations of certain helical residues reduce or even eliminate the channel responses induced by ligand binding (Trapp, Tucker & Aschroft, 1998a; Minor et al., 1999; Piao et al., 2001; Cui et al., 2003; Wu et al., 2004). However, it is unclear how the membrane helices act in channel gating. If the membrane helices are not a simple follower, they should be able to influence the consequence of ligand binding. An ultimate demonstration would be that channel activity is determined by the helical conformations, no matter whether the ligand normally is excitatory or inhibitory to the channel.

The Kir6.2 channel is inhibited by ATP and stimulated by proton (Davies, 1990; Davies et al., 1992; Inagaki et al., 1995; Xu et al., 2001). The Kir6.2 gating by ATP and proton requires specific membrane domains in Kir6.2, which cannot be substituted with those in Kir1.1, another member of the Kir family (Piao et al., 2001; Wu et al., 2004). Several residues in these membrane helices are important for the channel gating (Trapp et al., 1998a; Enkvetchakul et al., 2001; Piao et al., 2001; Cui et al., 2003; Wu et al., 2004). Among them are Thr71 and Cys166. When the Cys166 in the TM2 is mutated, the Kir6.2 gating by both ATP and proton is disrupted (Trapp et al., 1998a; Piao et al., 2001). The Thr71 is located at the boundary of the TM1 and N terminus. Full ATP and pH sensitivities require a residue with a flexible side-group at this site, while a bulky residue eliminates the ATP- and pH-dependent channel gating (Cui et al., 2003). According to the KirBacl.1 model (Fig. 1) (Kuo et al., 2003), Thr71 in the Kir6.2 channel is located in the immediate vicinity of Cys166, which is remarkable, as these residues' close location at the interface of the TM1 and TM2 may allow an interaction between these two membrane helices. Thus, the gating mechanism may be accessible for intervention by manipulation of these helices' interaction. Therefore, we studied the effect of TM1-TM2 interaction on Kir6.2 gating by placing opposite or identical charges at the 71 and 166 sites. Surprisingly, we found that the gating process of the Kir6.2 channel was completely reversed with opposite charges at these sites, making ATP an activator and proton an inhibitor of the channel.

Materials and Methods

Kir6.2 (GenBank accession #D50581) and Kir2.1 (#X73052) were used in the present study. The cDNAs were cloned to a eukaryotic

expression vector (pcDNA3.1, Invitrogen, Carlsbad, CA) and expressed in frog oocytes.

Frog (*Xenopus laevis*) oocytes were obtained as we described previously (Xu et al., 2001; Piao et al., 2001), and treated with 2 mg/ml of collagenase (Type F, Sigma, St Louis, MO) in an OR2 solution (in mM) NaCl 82, KCI 2, $MgCl_2$ 1 and N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid or 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 were injected into the oocytes (20–50 ng in 50 nl double-distilled water). The oocytes were then incubated at 18°C in an 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).

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 with the perfusion solution bathing both the top and bottom surface of the oocytes. There was a 3 mm × 15 mm gap on the top cover of the chamber, which served as the gas outlet and access to the oocytes for recording microelectrodes. The perfusate contained (mM): KCl 90, $MgCl_2$ 3, HEPES 5 (pH 7.4). At baseline, the chamber was ventilated with atmospheric air. Exposure of the oocytes to CO_2 was carried out by switching to a perfusate that had been bubbled for at least 30 min with a gas mixture containing 15% CO_2 balanced with 21% O_2 and N_2, and superfused with the same gas. The high dissolvability of CO_2 resulted in a detectable change in intra- or extracellular acidification as fast as 10 s in these oocytes. We have previously measured intra- and extracellular pH in *Xenopus* oocytes with various PCO_2 levels (Zhu et al., 2000), and used these values in our present studies.

To determine channel expression, whole-cell currents were studied on the oocytes 2–4 days after injection. Two-electrode voltage clamp was performed using an amplifier (Geneclamp 500, Axon Instruments, Foster City, CA) at room temperature (24–25°C). The extracellular solution contained (in mM): 90 KC1, 3 $MgCI_2$, and 5 HEPES (pH 7.4).

Patch-clamp experiments were performed at room temperature as described previously (Xu et al., 2001). In brief, fire-polished patch pipettes were made from 1.2 mm borosilicate glass capillaries using a Sutter P-97 puller (Sutter Instrument, Novato, CA). Giant inside-out patches were employed to study macroscopic currents in a cell-free condition using recording pipettes of 0.5–1.0 MΩ and an Axo-patch 200B amplifier (Axon Instruments). The current records were low-pass filtered (Bessel, 4-pole filter, -3 dB at 2 kHz), digitized with pClamp8.1 software (Axon Instruments), and stored on computer disk for later data analysis. 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.

The ATP-current relationship was expressed with the Hill equation: $y = 1/[1 + ([ATP] / IC_{50})^h]$, where [ATP] = ATP concentration, and IC_{50} = the [ATP] at midpoint channel inhibition. The dose-response relationship for pH sensitivity was similarly produced in Kir2.1 and its mutants. Data are presented as means ± SE. ANOVA was used to analyze the variations of data within the group. Differences in means were tested with Student's *t*-test and were accepted as significant if $P \leq 0.05$.

Results

Baseline Properties

Experiments were performed on the Kir6.2 with 36 amino acids truncated in the C terminus, i.e., Kir6.2ΔC36 and its mutant channels. We chose to use the Kir6.2ΔC36 for two reasons: 1) The Kir6.2ΔC36 can be expressed without the SUR subunit that is known to be involved in the channel gating (Tucker et al., 1997), and 2) like the wild-type (wt) channel, the Kir6.2ΔC36 is gated by ATP and pH (Wu et al., 2002). A bath solution (KD90) containing 90 mM K^+ was used to record whole-cell K^+ currents in two-electrode voltage clamp. These currents showed clear inward rectification with an amplitude of 2.1 ± 0.3 μA (n = 14 measured at –160 mV). They were inhibited by Ba^{++} and activated by azide (Piao et al., 2001; Wu et al., 2002). In contrast, oocytes that received an injection of the expression vector alone did not express such inward-rectifying currents. The small currents recorded (0.1–0.4 μA) were insensitive to Ba^{++} and azide.

The ATP sensitivity 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 membranes with command potentials from –100 mV to 100 mV (−150 mV to 150 mV in some cells) applied through the recording pipette. Under such a condition, the inward-rectifying currents showed a single-channel conductance of ~75 pS and were inhibited by ATP (K^+Salt) in a concentration-dependent fashion. The ATP concentration for 50% channel inhibition (IC_{50}) was ~100 μM.

The Kir6.2ΔC36 channel was strongly activated during an exposure to 15% CO_2, and this level of CO_2 causes intra- and extracellular acidifications (pH_i 6.6, pH_o 6.2) (Zhu et al., 2000; Xu et al., 2000), and augments the Kir6.2ΔC36 currents by ~130% (Xu et al., 2001; Piao et al., 2001). We have previously shown that pH_o has no effect on the Kir6.2 channel, and that the current augmentation is solely produced by intracellular acidification (Xu et al., 2001). To avoid channel rundown, which is constantly seen at acidic pH levels in excised patches (Xu et al., 2001; Wu et al., 2002), all pH experiments on the Kir6.2ΔC36 and its mutant channels were performed in whole-cell recordings using 15% CO_2.

Double Mutations Have Drastic Effects on Both ATP and pH Sensitiviity

A previous study shows that Cys166 is not susceptible to thiol-reactive agent (Trapp et al., 1998b). We also failed when we attempted to test the interaction by mutating the Thr71 to Cysteine and see whether it can form disulfide bond with Cys166. Subsequently, the interaction between these residues was studied by introducing charged amino acids to replace Thr71 and Cys166.

Joint mutations of Thr71 to lysine and Cys166 to glutamate produced functional currents that showed

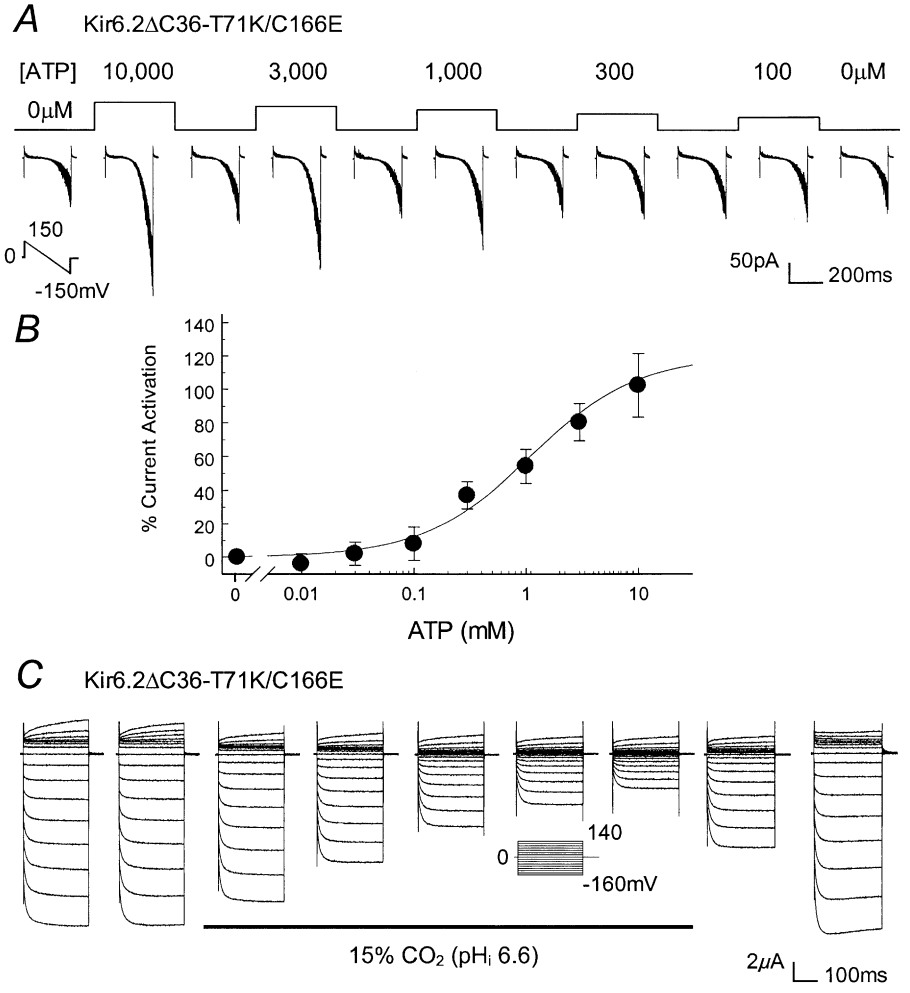

Fig. 2. Effects of ATP and pH on Kir6.2*Δ*C36 with T71K/C166E mutations. (*A*) Microscopic currents were recorded from a giant inside-out patch with 145 mM K^+ on both sides of the patch membrane. Perfusates with different concentrations of ATP were applied to the intracellular membrane for 1 minute. Ramp potentials from -150 mV to 150 mV are given at a holding potential of 0 mV. In contrast to the Kir6.2*Δ*C36 (Tucker et al., 1997), the T71K/C166E currents were stimulated by intracellular ATP in a concentration-dependent fashion. Note that eight superimposed traces are shown in each panel. (*B*) The dose-response curve is fitted with the Hill equation ($y = Im/(1+(EC_{50}/[ATP])^h)$, where [ATP] = ATP concentration; EC_{50} (1.1 mM) = is the ATP concentration for 50% current stimulation; h (0.9) = Hill coefficient; and Im = maximal current activation. (*C*) Whole-cell currents were studied in voltage clamp in an oocyte. The T71K/C166E currents were strongly inhibited by intracellular acidification produced with an exposure to 15% CO_2 (pH_i 6.6) for 6 min.

clear inward rectification in the absence of exogenous polyamines with a single-channel conductance of 74.4 ± 1.3 pS (n = 8). The open-state probability (P_o = 0.099 ± 0.023, n = 6) was ~4-fold higher than that of Kir6.2*Δ*C36. Surprisingly, we found that the T71K/C166E currents were no longer inhibited by ATP. Instead, the currents were strongly augmented by intracellular ATP (Fig. 2*A*). The channel activity increased by 181.0 ± 9.2% (n = 9) in the presence of 3 mM ATP. The dose-response relationship showed the EC_{50} concentration of ATP to be ~800 μM (Fig. 2*B*). This channel activation is not produced by protein phosphorylation, as the non-hydrolyzable ATP analog 5′-adenylyl β,y-imidodiphosphate (AMP-PNP, 3 mM) had a similar effect (202.5 ± 12.9%, 12.9%, n = 4; $P > 0.05$). Thus, these mutations make ATP an activator of the Kir6.2*Δ*C36 channel.

Consistent with the ATP result the pH effect on whole-cell T71K/C166E currents was also completely reversed. The baseline T71K/C166E currents were much larger (13.4 ± 2.8 μA, n = 7) than those of Kir6.2*Δ*C36 (2.0 ± 0.5 μA, n = 16). These currents were strongly inhibited when the cells were exposed to 15% CO_2 (Fig. 2*C*). The inhibition took place within 1 min, reached a plateau in 3–4 min, and was fully reversible with washout (Fig. 2*C*). Repetitive exposures to CO_2 had the same effect. Similar reversal of the ATP and pH sensitivities was observed in the T71R/C166E mutant (Fig. 3*A*, *C*, *D*, Table 1), suggesting that the effects are likely to be produced by the electrostatic interaction between these two sites.

When these residues were mutated to alternative sets of oppositely charged amino acids (T71E/C166K,

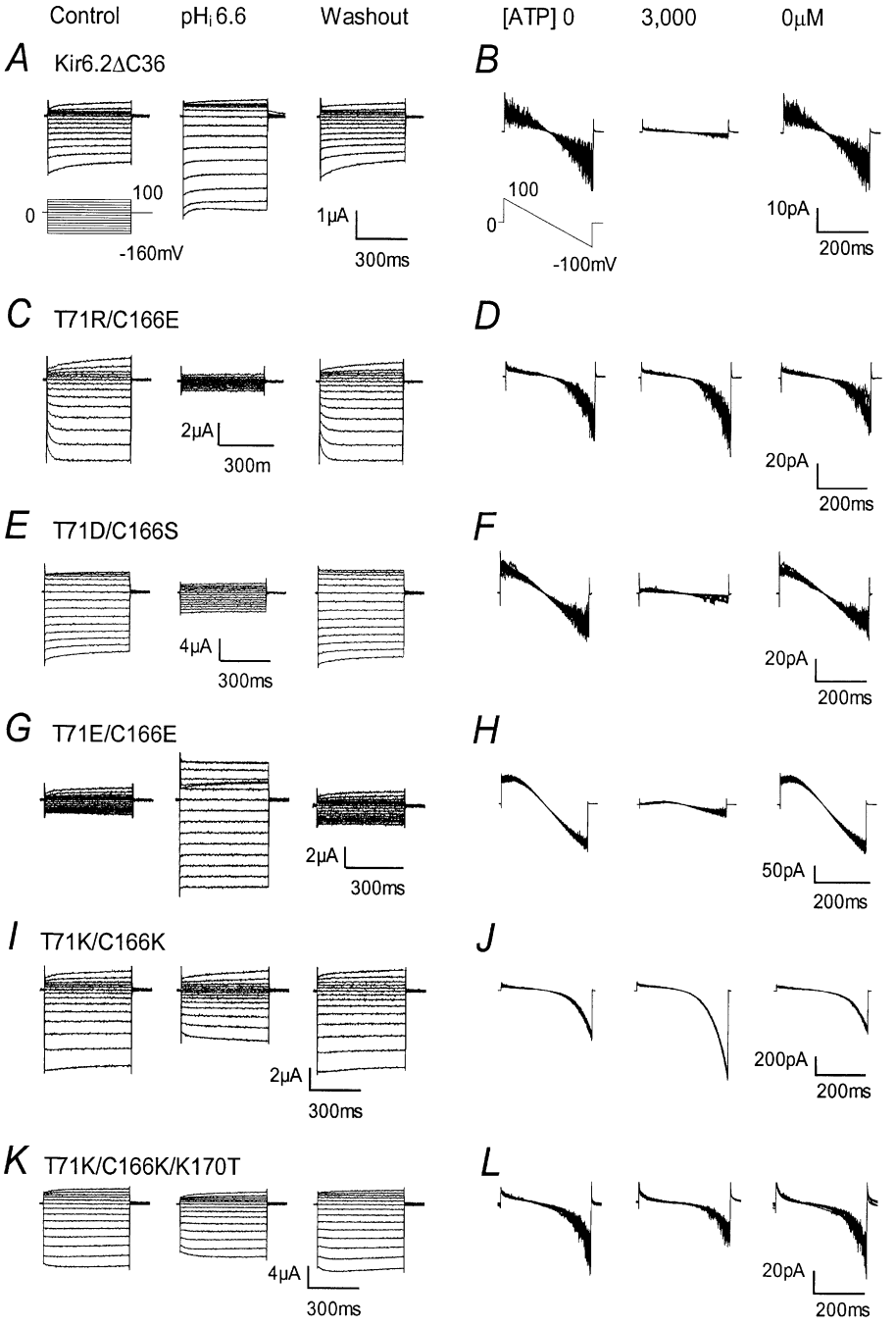

Fig. 3. Responses of several Kir6.2ΔC36 mutants to ATP and acidic pH. Channel responses to pH and ATP were studied as in Fig. 2*A, C*. (*A*, *B*) The Kir6.2ΔC36 channel was stimulated by hypercapnic acidosis and inhibited by 3 mM ATP. (*C, D*) The T71R/C166E was inhibited by acid pH, and modestly stimulated by 3 mM ATP. (*E*, *F*) The pH but not ATP sensitivity was reversed in the T71D/C166S mutant. (*G*, *H*) The T71E/C166E currents were strongly augmented by low pH, and inhibited by ATP. (*I*, *J*) The pH and ATP sensitivities were both reversed in the T71K/C166K. (*K*, *L*) By adding a third mutation, the T71K/C166K/K170T was inhibited by both ATP and acidic pH. Note that all records for the ATP sensitivity are shown with eight superimposed traces.

T71D/C166K, and T71E/C166R), none of the mutant channels expressed detectable currents. However, the T71D/C166S mutant produced inward-rectifying currents, in which a reversal of the pH- but not ATP-sensitivity was observed (Fig. 3*E*,*F*). Since such a reversal of ATP and pH sensitivities was not seen in individual mutation of either Thr71 or Cys166 (Table 1), these results suggest that the manipulation of these two residues may have restructured the gating mechanism.

To create a repulsive force between these two sites, both Thr71 and Cys166 were mutated to

Table 1. List of all wild-type and mutant channels studied and their ATP and pH sensitivities

Name	IC_{50} ATP (μM)	pK_a	pH_i effect (%)	BL Current (μA)
Kir6.2ΔC36	109 ± 10(9)	–	128.6 ± 11.4(16)	2.0 ± 10.5(16)
Kir6.2 ± a SUR1	6 ± 1 (5)	–	131.4 ± 125.2(5)	2.2 ± 3.1 (5)
Kir2.1	–	4.96 ± 0.01 (6)	2.81 ± 1.1 (6)	16.3 ± 5.0(6)
Kir6.2 mutant				
T71E	740 ± 73 (5)	–	44.0 ± 11.1 (11)	20.4 ± 4.7(11)
T71K	600 ± 47 (5)	–	−13.9 ± 4.2 (12)	2.4 ± 0.3(12)
C166E	2,500 ± 188(4)	–	17.1 ± 16.7(8)	18.1 ± 1.5(8)
C166K	NF	NF	NF	NF
C166S	>10,000	–	−0.5 ± 1.8 (6)	18.7 ± 5.0(6)
T71K/C166E	EC_{50}829 ± 125(9)	–	−78.9 ± 5.2 (7)	13.4 ± 2.8(7)
T71R/C166E	EC_{50}6,500 ± 1,323(5)	–	−79.9 ± 5.7 (4)	10.4 ± 2.8(4)
T71E/C166K	NF	NF	NF	NF
T71D/C166K	NF	NF	NF	NF
T71E/C166R	NF	NF	NF	NF
T71E/C166K/K170T	NF	NF	NF	NF
T71D/C166S	361 ± 18 (5)	–	−61.0 ± 3.6(6)	7.0 ± 1.1 (6)
T71E/C166E	1,059 ± 2,98 (6)	–	765.2 ± 93.4 (6)	3.5 ± 0.5 (6)
T71D/C166E	NF	–	NF	NF
T71K/C166K	EC_{50} 2,080 ± 462 (7)	–	−41.5 ± 5.0 (11)	8.4 ± 0.3 (11)
T71K/C166K/K170T	>8,000 (5)	–	−17.3 ± 4.1 (8)	14.8 ± 2.5 (8)
T71E/C166E/K170T	NF	NF	NF	NF
Kir2.1 mutant				
M84K	–	6.84 ± 0.02 (4)	−50.0 ± 4.3 (14)	5.5 ± 1.3 (14)
M84E	–	–	−4.9 ± 5.5 (10)	18.2 ± 3.9 (10)
A178E	–	–	23.1 ± 15.9 (7)	3.7 ± 1.0 (7)
M84E/A178E	–	7.14 ± 0.05 (5)	263.3 ± 36.7 (6)	1.8 ± 0.2(6)
M84K/A178E	NF	NF	NF	NF
M84D/A178S	NF	NF	NF	NF
M84E/A178S	–	6.44 ± 0.05 (4)	−14.0 ± 2.4 (6)	10.7 ± 2.3 (6)
M84E/A178S/K182N	NF	NF	NF	NF
M84K/A178D	NF	NF	NF	NF

The ATP sensitivity was studied in excised patches and is expressed by fitting the data using the Hill equation shown in Fig. 2*B*. Hill coefficients are 0.9–1.2 (*not shown*) with *n* shown in the parenthesis. The pH sensitivity was studied in whole-cell recording using 15% CO_2 (pH_i 6.6) and is expressed as % change in the current amplitude. All Kir6.2 mutants were created with the Kir6.2ΔC36. *Abbreviations*: *BL*, whole-cell baseline; *NF*, nonfunctional.

identically charged residues. The T71E/C166E mutant expressed inward-rectifying currents. This mutant was inhibited by ATP and stimulated by hypercapnic acidosis (Fig. 3*G*, *H*). Although its ATP sensitivity was lower than that of the *wt* channel, the stimulatory effect of pH was much greater (Table 1, Fig. 4*B*). With two positive charges at these locations, the T71K/C166K was inhibited by proton and stimulated by ATP, a phenotype that is more like the T71K/C166E than the T71E/C166E (Fig. 3*I*, *J* and Fig. 4*A*, *B*). According to the KirBac1.1 model (Fig. 1)(Kuo et al., 2003), there is a basic residue (Lys170) at the surrounding area of Thr71 and Cys166. Since the Lys170 is situated at just one helical turn below Cys166 and also faces Thr71, it is possible that the Lys170 is also involved in the newly created electrostatic interactions between the TM1 and TM2 residues. Thus, we additionally mutated the Lys170 to a neutral threonine. The inhibitory effect of lowering pH on the T71K/C166K/K170T was significantly smaller (−17.3 ± 4.1%, $n = 18$) than that on the T71K/C166K (−41.5 ± 5.0%, $n = 11$; $P < 0.01$) (Fig. 3*K*, *L*). More importantly, the T71K/C166K/K170T was inhibited, but not stimulated, by ATP (Fig. 4*A*, Table 1), further suggesting that the opening and closure of the Kir6.2 channel can be predetermined by strengthening or weakening the interaction of the TM1 with TM2 helices.

Similar Effects Were also Observed in the Kir2.1 Channel

To elucidate whether the electrostatic interactions at these two locations affect the gating process of other Kir channels, we performed similar studies on the Kir2.1 channel known to be insensitive to pH (Fakler et al., 1996; Zhu et al., 1999; Qu et al., 2000) (Fig. 5*A*). With identical charges at sites corresponding to Thr71 and Cys166 in Kir6.2, the M84E/A178E was strongly stimulated by acidic pH (Fig. 5*B*), consistent with the T71E/C166E mutant in Kir6.2. Although the M84K/A178E and M84E/A178K mutations did not yield functional channels,

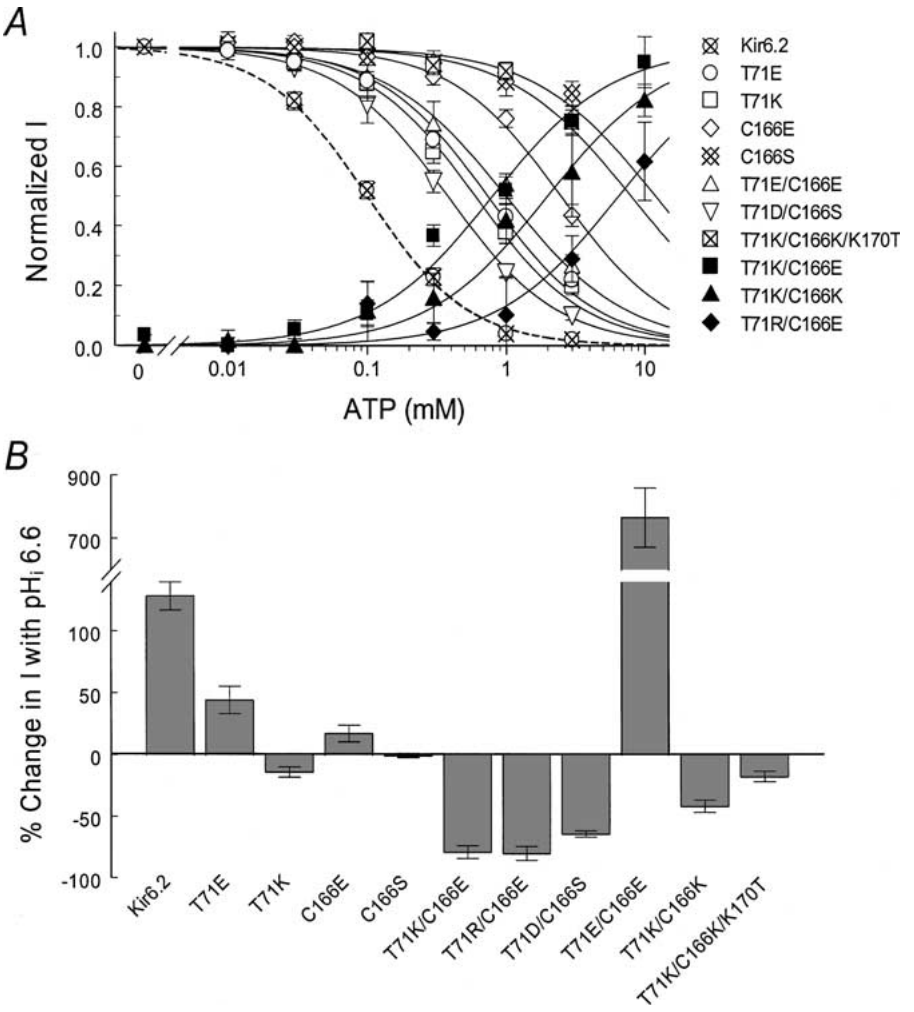

Fig. 4. ATP and pH sensitivities of Kir6.2ΔC36 mutants. (*A*) Dose-response relationship of Kir6.2ΔC36 mutants was studied using the Hill equation as shown in Fig. 2*B*. Whereas most mutant channels remained inhibited by intracellular ATP, T71K/C166E, T71R/C166E and T71K/C166K were augmented *See* Table 1 for the IC_{50} and EC_{50}. The h = 0.9–1.2; n (number of patches) = 4–9. (*B*) The pH sensitivity was studied using 15% CO_2 as shown in Fig. 2*C*. The T71K/C166E, T71R/C166E T71D/C166S and T71K/C166K were markedly inhibited at pH_i 6.6. In contrast, the T71E/C166E was strongly stimulated at this pH level.

the M84E/A178S expressed inward-rectifying currents. Exposure to hypercapnic acidosis caused inhibition of the M84E/A178S mutant (Fig. 5*C*). The pH-current relationship was studied using inside-out patches. The dose-response curves of these two mutants were exactly opposite to each other (Fig. 6). Thus, the gating mechanism in the Kir2.1 channel can also be rearranged by changing the electrostatic interactions at these two sites.

Discussion

Recent studies suggest that membrane helices may play a more active role in the channel gating. The opening and closure of the two-pore chloride channel, gap junction channels, and the influenza virus M2 proton channel are determined by the side-chain movement of a few pore-lining residues (Bennett et al., 1991; Tang et al., 2002; Dutzler, Campbell & Mackinnon 2003). The gating of the bacterial mechanosensitive (MscL) channel involves concerted movements of membrane helices and the side-group of two phenylalanine residues in the S1 domain (Sukharev et al., 2001). It is known that the membrane helices of the ligand-gated and also perhaps of the voltage-gated channels change their conformations during gating (Yellen et al., 2002; Kuo et al., 2003). TM1 and TM2 helices undergo a counter-clockwise rotation after ligand binding. The narrowest part of the TM2 is widened. The opening of the channels allows certain ions and organic molecules to access the inner cavity (Armstrong et al., 1966; Liu et al., 1997; del Camino et al., 2000; Enkvetchakul et al., 2001; Flynn et al., 2001; Shin et al., 2001; Jin et al., 2002; Phillips et al., 2003; Perozo, Cortes & Cuello,

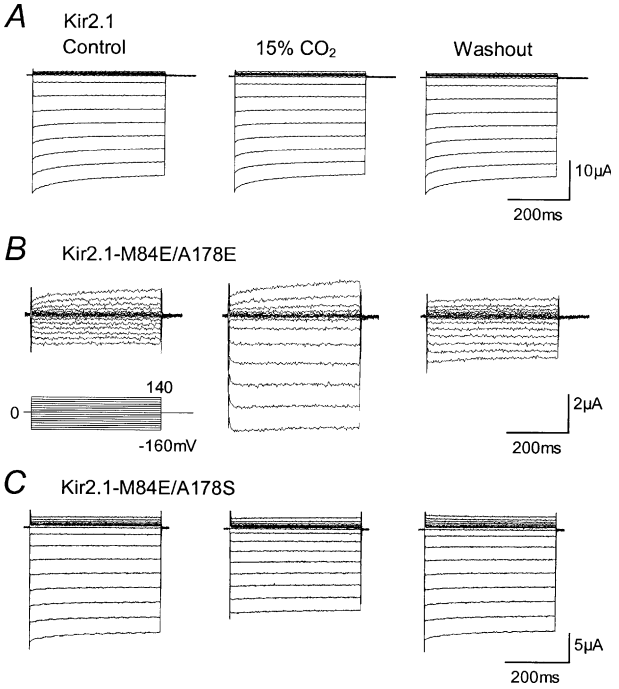

Fig. 5. The effect of acidic pH on Kir2.1 mutants. Intracellular acidification was produced using 15% CO_2 and channel response was studied as in Fig. 2*C*. (*A*) The wt Kir2.1 channel was unaffected by 15% CO_2. (*B*) The M84E/A178E mutant had small baseline currents. This channel was strongly augmented by 15% CO_2. (*C*) In contrast, the M84E/A178S mutant was inhibited.

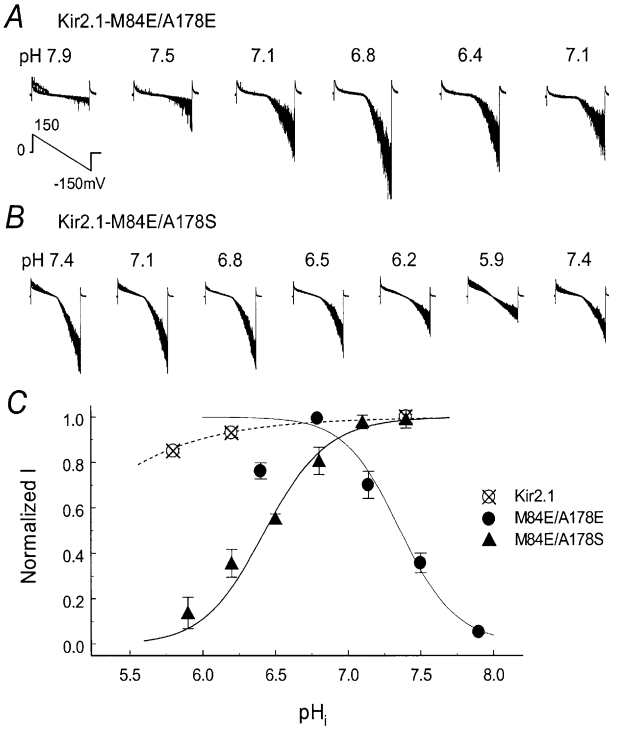

Fig. 6. The pH sensitivity of Kir2.1 mutants studied in inside-out patches. (*A*) the Kir2.1-M84E/A178E currents were rather small at pH 7.9. Reduction of pH levels in the internal solution produced graded augmentation of the Kir2.1-M84E/A178E currents. The maximal channel activation is reached at pH 6.8. The currents started being inhibited at pH 6.4. (*B*) The Kir2.1-M84E/A178S mutant showed rather large currents at baseline (pH 7.4), and was inhibited with graded reductions in pH. (*C*) The dose-response relationship of these two mutants was expressed using the Hill equation. The Kir2.1-M84E/A178E currents were half activated at pH 7.14 ($n = 5$), and the Kir2.1-M84E/A178S was half inhibited at pH 6.40 ($n = 4$).

1999). Our current studies present another novel finding, namely, that the gating mechanism is not fixed in Kir channels but can, indeed, be reset by manipulating electrostatic interaction between the TM1 and TM2.

Based on the KirBac1.1 model (Kuo et al., 2003), the TM1 crosses the TM2 at the inner pore region, where Thr71 and Cys166 are found in the Kir6.2 channel. Our results clearly show that electrostatic forces introduced between these two sites dramatically affect the channel gating, suggesting that the TM1 and TM2 helices in Kir6.2 channels do not act independently. In contrast, they appear to interact with each other, determining the consequence of ligand binding. It is likely that the electrostatic interaction causes a change in the relative distance of the TMs and perhaps their membrane position as well. Such a change may assign a new conformation to the gating assembly, affecting its operation by ligand binding. We believe that the Kir6.2 channel gating by ATP and proton depends on specific movements of the TM2 and TM1, including rotation, anti-parallel sliding and lateral movement (Perozo et al., 1999; del Camino et al., 2000; Johnson & Zagotta, 2001; Schulte et al., 2001; Jiang et al., 2002; Jin et al., 2002; Dutzler et al., 2003). Such conformational changes are interfered with or disrupted by the additional electrostatic forces between the membrane helices. The T71K/C166E and T71R/C166E mutations switch the movement to the opposite direction and reverse the gating process. With the T71E/C166E mutation, the helical movements are slightly altered, so that the mutant still responds to ATP and pH similarly to the *wt* channel. This phenomenon is not only limited to the Kir6.2 channel, as we have observed channel activation and inhibition by acidic pH in the Kir2.1 channel by similar manipulations of corresponding residues. It is worth noting that the close location of Thr71 to Cys166 is predicted with the KcsA and KirBac1.1 models. Their relative locations may be different in the open state since both these channels were crystallized in their closed state.

It is possible that Kir channel gating involves multiple interactions between TM1 and TM2 residues, as there is evidence showing that specific protein domains and amino-acid residues in the membrane helices are required for channel gating. A

single amino-acid mutation can produce effects from modest reduction to complete loss of the gating as shown in the channel sensitivity to intracellular ligand molecules (Trapp et al., 1998a; Enkvetchakul et al., 2001; Piao et al., 2001; Cui et al., 2003; Wu et al., 2004). Since both TM1 and TM2 undergo rotation during channel gating (Perozo et al., 1999), one residue may face and interact with different amino acids on the other TM domain. In our study, the Lys170 close to the Cys166 at just one helical turn below is also involved in the electrostatic interaction, as the T71K/C166K/K170T is gated differently from the T71K/C166K. With three positive charges, the T71K/C166K mutant behaves more like the T71K/C166E. How this occurs is unclear. The interaction may involve residues beyond these three amino acids, which we cannot answer due to difficulties to explore all potential interactions. We believe that the Thr71 plays a key role in the multiple interactions because most mutants with a lysine at this position respond to ATP and pH similarly. However, the dominant effect of Thr71 should not compromise the contribution of Cys166 to the channel gating, as the gating of T71K mutant is not totally reversed. The channel inhibition by acidic pH is unlikely to be produced by titration of the newly created lysine residue, because the T71K/C166E and T71D/C166S are gated similarly by pH, and because the ATP sensitivity is also reversed. Although there are still unexplained phenomena, our studies have shown for the first time that the TM1 and TM2 helices can interact with each other, and the Kir channel gating can be intervened with by their interaction at the inner pore region.

Beyond the consistency with previous findings that both membrane helices participate in channel gating, our results strongly suggest that the membrane helices are not simply followers of the intracellular ligand-binding domains whose conformation ultimately governs the position and movement of the membrane helices, as shown in the bacterial MthK channel (Jiang et al. 2002). These helices in the mammalian Kir6.2 channel seem to be sufficient to determine the channel opening or closure following binding to the same ligand, depending on how they interact with each other in their local environment.

In conclusions, the Kir6.2 gating involves Thr71 in the TM1 and Cys166 in TM2. Creation of electrostatic attraction at these sites completely reverses the channel gating by ATP and proton. Similar results were observed in the pH-dependent gating of the Kir2.1 channel. Thus, the membrane helices do not seem to act as followers of the C terminus in these mammalian K^+ channels, and they instead may determine whether these channels are open or closed following ligand binding to the channel protein.

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