J Membrane Biol (2007) 215:181–193
DOI 10.1007/s00232-007-9017-0

Charges in the Cytoplasmic Pore Control Intrinsic Inward Rectification and Single-Channel Properties in Kir1.1 and Kir2.1 Channels

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Received: 29 January 2007 / Accepted: 27 February 2007 / Published online: 14 June 2007

Abstract An E224G mutation of the Kir2.1 channel generates intrinsic inward rectification and single-channel fluctuations in the absence of intracellular blockers. In this study, we showed that positively charged residues H226, R228 and R260, near site 224, regulated the intrinsic inward rectification and single-channel properties of the E224G mutant. By carrying out systematic mutations, we found that the charge effect on the intrinsic inward rectification and single-channel conductance is consistent with a long-range electrostatic mechanism. A Kir1.1 channel where the site equivalent to E224 in the Kir2.1 channel is a glycine residue does not show inward rectification or single-channel fluctuations. The G223K and N259R mutations of the Kir1.1 channel induced intrinsic inward rectification and reduced the single-channel conductance but did not generate large open-channel fluctuations. Substituting the cytoplasmic pore of the E224G mutant into the Kir1.1 channel induced open-channel fluctuations and intrinsic inward rectification. The single-channel conductance of the E224G mutant showed inward rectification. Also, a voltage-dependent gating mechanism decreased open probability during depolarization and contributed to the intrinsic inward rectification in the E224G mutant. In addition to an electrostatic effect, a close interaction of K^+ with channel pore may be required for generating open-channel fluctuations in the E224G mutant.

Keywords Intrinsic inward rectification · Kir channel · Electrostatic potential · Single-channel conductance · Open-channel fluctuation · Chimera

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Introduction

Inwardly rectifying K^+ (Kir) channels are important in maintaining stable resting membrane potentials and controlling the excitability of many cell types. The physiological functions of strong inwardly rectifying channels are related to their unique inward rectification, which allows K^+ influx but limits K^+ efflux (Hille, 2001). In the cloned Kir2.1 channel (Kubo et al., 1993), the mechanism underlying this inward rectification has been attributed to the voltage (V_m)-dependent block caused by intracellular Mg^{2+} and polyamines interacting with residues D172 (Ficker et al., 1994; Kubo & Murata, 2001; Lopatin, Makhina & Nichols, 1994; Lu & MacKinnon, 1994; Matsuda, Saigusa & Irisawa, 1987; Vandenberg, 1987; Yang, Jan & Jan, 1995). In addition to D172, several negatively charged residues located in the cytoplasmic pore have been shown to be involved in interacting with polyamines and Mg^{2+} and, thus, to induce inward rectification. It has been shown that the neutralization of E224, E299, D255 or D259 decreased the V_m-dependent block of the Kir2.1 channel by internal polyamines and Mg^{2+} (Kubo & Murata, 2001; Pegan et al., 2005; Yang et al., 1995). Recently, it has been found that the intensity of inward rectification induced by V_m-dependent block is determined not just by the negatively charged residues. A decrease in the net negative charge within the cytoplasmic pore by replacing R228 or R260 with a neutral residue reduces the V_m-dependent block by internal Mg^{2+} and polyamines (Fujiwara & Kubo, 2006). The role of the net negative charges in the cytoplasmic pore is proposed to accumulate K^+ and internal blockers to facilitate K^+ permeation and transport the blockers to the deeper channel blocking site (Fujiwara & Kubo, 2006; Kubo & Murata, 2001).

Previous studies suggest that, independent of channel block, intrinsic inward rectification is present in the Kir2.1 channel (Ishihara et al., 1989; Matsuda, Oishi & Omori, 2003; Shieh et al., 1996). Also, replacement of D172 with lysine, histidine or arginine results in inward rectification that is independent of channel block, and the effect is attributed to an intrinsic inward rectification (So et al., 2003). Furthermore, E224G and E224Q mutants show stronger degrees of inward rectification in the absence of internal blockers (Fujiwara & Kubo, 2006; Xie et al., 2004; Yeh, Chang & Shieh, 2005). These studies suggest that the charge at site 224 contributes to a local electrostatic potential and regulates the intrinsic inward rectification. In addition to the charge at site 224, other charged residues near site 224 are involved in the intrinsic inward rectification. It has been shown that the intensity of the intrinsic inward rectification of the E224Q mutant is reduced by the H226E (Xie et al., 2004) and R228Q (Fujiwara & Kubo, 2006) mutations. Also, the amino acid sequence of the Kir1.1 channel is similar to that of the Kir2.1 channel (Kubo et al., 1993). The residue equivalent to E224 in the Kir2.1 channel is a glycine (G223) in the Kir1.1 channel (Ho et al., 1993), which, in contrast to the E224G mutant of the Kir2.1 channel, does not show inward rectification in the absence of intracellular blockers. These results further suggest that the lack of a negative charge at residue 224 is not a necessary condition for the intrinsic inward rectification. The structure of a Kir2.1L, which contains the N-terminal domain (residues 41–64) fused to the entire C-terminal domain (residues 189–428) of a mouse Kir2.1 channel, shows that sites H226, R228 and R260 are positively charged, located close together and face the aqueous pore in the Kir2.1 channel (Fujiwara & Kubo, 2006; Pegan et al., 2005) (Fig. 1). In the Kir1.1 channel, the amino acids at the equivalent positions (H225, Y227 and N259) are also located close by, but two of them are not charged (Fig. 1).

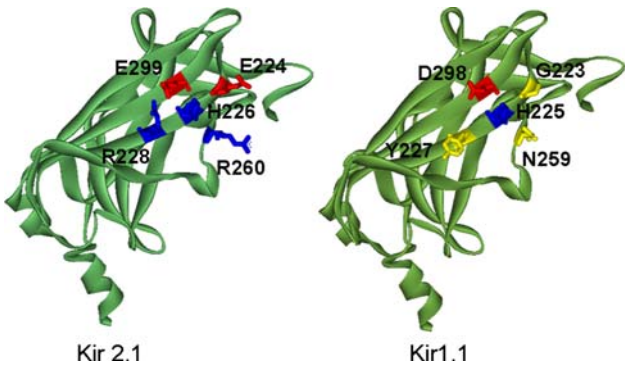

Fig. 1 Structural models of Kir2.1 and Kir1.1 channels constructed based on the Kir2.1L structure. The cytoplasmic pore of one subunit is shown. Charged residues lining the channel pore are shown in ball-and-chain models. *Blue* denotes a positively charged residue and *red*, a negatively charged residue

Although the effects of various mutations on the block of the Kir2.1 channel by internal Mg^{2+} and polyamines have been extensively studied (Fujiwara & Kubo, 2006), only the effects of H226E, D259N and R228Q on the intrinsic inward rectification have been examined (Fujiwara & Kubo, 2006; Xie et al., 2004).

The explanation that the effects of charge mutations are due to the electrostatic potential at and near E224 is based on a limited amount of mutations rather than systematic mutations. Also, previous studies have not determined the gating mechanism underlying the electrostatic effects causing intrinsic inward rectification. For example, the intrinsic inward rectification in the E224G mutant has not been examined at the single-channel level. It is unknown whether the intrinsic inward rectification is due to a V_m-dependent gating or V_m-dependent conductance. In E224G and E224Q mutants, single-channel currents show open-channel fluctuations in the absence of polyamines and Mg^{2+} (Fujiwara & Kubo, 2006; Kubo & Murata, 2001; Xie et al., 2004; Yang et al., 1995). However, in the Kir1.1 channel, site 223 (equivalent to site 224 of Kir1.1) is also a glycine, but the single-channel currents do not display open-channel fluctuations. The mechanism underlying the differences in single-channel properties and intrinsic inward rectification between the E224G mutant and Kir1.1 channel remains unknown. In this study, we examined the effects of positively charged residues (H226, R228 and R260) near site 224 on the intrinsic inward rectification and single-channel properties of the E224G mutant of the Kir2.1 channel. The results showed that the greater number of positively charged residues around residue 224 in the E224G mutant of the Kir2.1 channel than around G223 in the Kir1.1 channel makes the former more strongly rectifying. Also, in addition to the electrostatic effect, the open-channel fluctuations observed in the E224G mutant probably require the additional close interaction of permeant K^+ ions with the cytoplasmic pore. Our results show that the intrinsic inward rectification of the E224G mutant is regulated by both V_m-dependent ion conductance and a V_m-dependent gating mechanism controlling the transitions between the closed state, substate and main open state.

Materials and Methods

Molecular Biology and Preparation of *Xenopus* Oocytes

Mutations were constructed using polymerase chain reaction (PCR) and checked by sequencing. Kir1.1 and Kir2.1 cDNAs with 5′- and 3′-untranslated regions were subcloned into the pGEMHE vector. The $Kir1.1_{1–183}$-$Kir2.1_{185–428}$ chimera was constructed by PCR amplification. *Eco*R V cloning sites were silently introduced at S183 of the Kir1.1

and at A184 of the Kir2.1 channel. Both the fragment of Kir2.1 COOH terminus (amino acids 185–428) and the fragment of pGEMHE-Kir1.1 (amino acids 1–183) were amplified by primers containing mutated *Eco*R V and original *Pst*I restriction sites. Subsequently, the two fragments were digested with *Eco*R V and *Pst*I and then ligated. The chimera was then sequenced to verify the correct chimerical construction, and the sequence at the ligating site is $L_{179}A_{180}K_{181}I_{182}S_{183}$-$K_{185}P_{186}K_{187}K_{188}R_{189}$. The cRNAs were obtained by *in vitro* transcription (mMessage mMachine; Ambion, Dallas, TX). *Xenopus* oocytes were isolated by partial ovariectomy from toads anesthetized with 0.1% tricaine (3-aminobenzoic acid ethyl ester). The incision was sutured and the animal monitored during the recovery period before it was returned to its tank. Following the final oocyte collection, the toads were anesthetized as described above and killed by decapitation. All surgical and anesthetic procedures were reviewed and approved by the Academia Sinica Institutional Animal Care and Utilization Committee. Oocytes were maintained at 18°C in Barth's solution, containing (in mM) NaCl 88, KCl 1, $NaHCO_3$ 2.4, CaN_2O_6 0.3, $CaCl_2$ 0.41, $MgSO_4$ 0.82 and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 15 (pH 7.6), with gentamicin (20 μg/ml), and were used 1–3 days after cRNA injection.

Electrophysiology

Giant-patch and single-channel recordings were sampled at 10–20 kHz and filtered at 2–5 kHz using the patch-clamp technique (Hamill et al., 1981; Hilgemann, 1995) and an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) at room temperature (21–24°C). The resistance of the electrodes ranged 1–3 MΩ for single-channel recordings and 0.15–0.25 MΩ for giant-patch recordings when filled with a 100 mM K^+ solution. The internal and external solutions consisted of (in mM) KCl 62, KOH 20, ethylenediaminetetraacetic acid (EDTA) 5, K_2HPO_4 8 and KH_2PO_4 2 (pH 7.4). The V_m command and data acquisition were performed using a Pentium-based personal computer, a DigiData board and pClamp6 software (Axon Instruments). For macroscopic current recordings, patches were held at 0 m*V*, prepulsed to –80 m*V* for 12 ms then stepped to test V_ms ranging from –200 to +100 m*V* for 20 ms. Capacitive and leak currents were corrected by subtracting the currents recorded after complete channel rundown from those measured during channel activity.

Data Analysis

Macroscopic instantaneous (peak) currents were measured at the beginning of the test V_ms. All single-channel current amplitudes except that of the E224G and Kir1.1$_{1–183}$-Kir2.1$_{185–428}$-E224G mutants were determined by half-amplitude threshold analysis using Clampfit. The single-channel current amplitude and kinetics of the E224G and Kir1.1$_{1–183}$-Kir2.1$_{185–428}$-E224G mutants were analyzed by mean-variance histograms (Chang, Yeh & Shieh, 2005). Averaged data are presented as the mean ± standard error of the mean (SEM). Student's independent *t*-test was used to assess statistical significance.

Results

Substituting Negatively Charged or Neutral Residues Near Residue 224 in the Kir2.1 Channel Corrected the Inward Rectification Induced by the E224G Mutation

According to the models (Fig. 1), three positively charged residues (H226, R228 and R260) are located near residue 224 in the Kir2.1 channel, but only one (H225) is found near residue 223 in the Kir1.1 channel. Previously, it was shown that, in the absence of intracellular blockers, an E224Q mutant of the Kir2.1 channel is more inwardly rectifying than the wild-type Kir2.1 channel and that the H226E and R228Q mutations can completely and partially, respectively, restore the *I-V* relationship of the E224Q mutant to that of the wild-type Kir2.1 channel (Fujiwara & Kubo, 2006; Xie et al., 2004). We first generated mutants in which a positively charged residue at residue 226, 228 or 260 was replaced with a glutamate to examine whether charge reversal around E224 affected the inward rectification of the E224G mutant. Figure 2 shows the macroscopic currents and peak *I-V* relationships of the wild-type channel and its E224G, E224G/H226E, E224G/R228E and E224G/R260E mutants. The degree of intrinsic inward rectification was greater in the E224G mutant than the wild-type Kir2.1 channel. Substituting a negative charge at residue 226, 228 or 260 increased the outward K^+ permeation and corrected the intrinsic inward rectification induced by the E224G mutation.

To determine whether the effect of the R260E mutation on the intrinsic inward rectification of the E224G mutant was due to the negative charge of the glutamate residue, we carried out other substitutions by replacing R260 in the E224G mutant with amino acids of various sizes, charges and polarities. Figure 3a shows the currents of mutants containing E224G/R260E and E224G/R260D (negative charge at site 260), E224G/R260A (small and nonpolar), E224G/R260W (large and nonpolar), E224G/R260G (small and polar), E224G/R260Q (large and polar), E224G/R260K (positive charge) and E224G/R260H (positive charge at low pH_i). Figure 3b shows that the normalized *I-V* relationships of the E224G/R260E and E224G/R260D mutants were similar to that of the wild-type Kir2.1

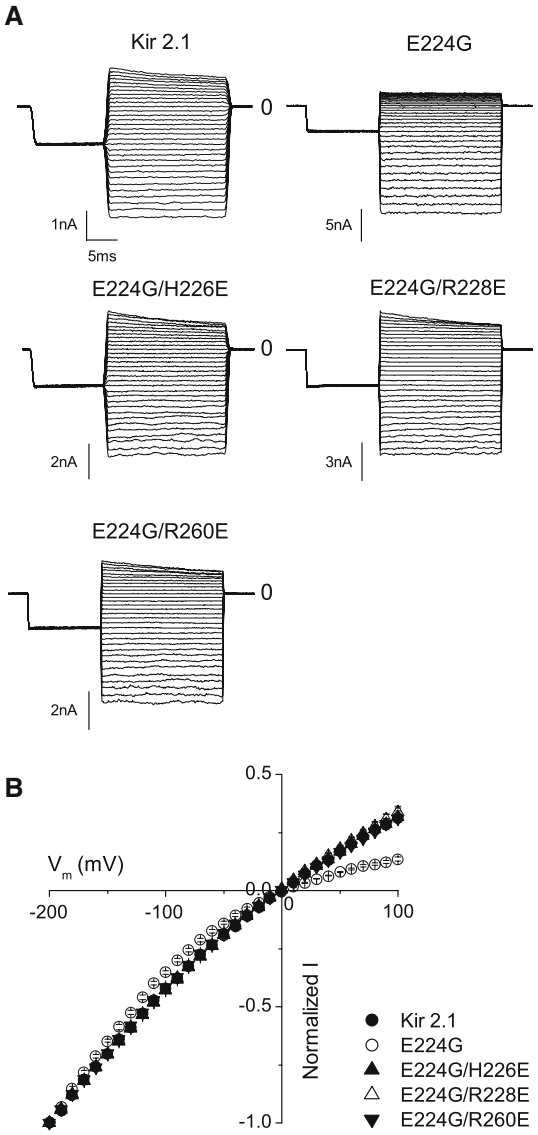

Fig. 2 Substituting glutamate at residue 226, 228 or 260 completely corrected the intrinsic inward rectification of the E224G mutant. (**a**) Macroscopic currents recorded from inside-out patches containing the wild-type Kir2.1 channel and its E224G, E224G/H226E, E224G/R228E and E224G/R260E mutants. Throughout this study, zero indicates zero current level. (**b**) Normalized *I-V* relationships of the indicated channels. Currents were normalized to that at –200 m*V* throughout this study. n = 3–5

channel. Neutralization of residue 260 so that the net charge of the double mutant was the same as that of the wild-type channel also corrected the inward rectification regardless of the polarity or size of the side chain (A, W, G or Q), and the normalized *I-V* relationships of these double mutants were the same as that for the wild-type channel (Fig. 3b). Replacing R260 with a K or H could not correct the inward rectification of the E224G mutant at pH_i 7.4. Figure 3c shows that the intrinsic inward rectification of the E224G/R260H mutant was stronger at a pH_i 6.0 than at pH_i 7.4 and was completely corrected at pH_i 9.0. The result is consistent with the hypothesis that when site 260 is more positively charged, the intrinsic inward rectification of the E224G mutant is stronger. Note that site H226 is also sensitive to internal pH_i and the intrinsic inward rectification of the E224G mutant is stronger when H226 is protonated (Xie et al., 2004). Therefore, the pH_i effects on the E224G/R260H mutants are mostly likely due to the protonation and deprotonation effects on both histidine residues at sites 226 and 260. We also examined the effects of pH_i on the intrinsic inward rectification of E224G. Figure 3c shows that when pH_i increased, the degree of intrinsic inward rectification decreased in the E224G mutant. Also, the degree of inward rectification was smaller in the E224G/R260H mutant than in the E224G mutant at pH 7.4, suggesting that pH_i affects both histidines at sites 226 and 260. In summary, the results suggest that, when the net charge of residues 224, 226, 228 and 260 is +3, the intrinsic inward rectification is stronger than that of a net charge of +1 and +2. If the charges at and around residue 224 contribute to a local electrical energy barrier responsible for inward rectification (Yeh et al., 2005), the charges in the cytoplasmic pore should have an additive effect. However, a net charge of +2 or +1 at the region near residue 224 in the E224G mutant had the same effect on the intrinsic inward rectification. It is possible that the intrinsic inward rectification of the E224G mutant is only weak and that a net charge of +2 is enough to completely correct the inward rectification. Consistent with this possibility, it has been previously shown that R228Q was unable to completely remove the intrinsic inward rectification of the E224Q mutant (Fujiwara & Kubo, 2006), which shows stronger intrinsic inward rectification than the E224G mutant.

If the charges around site 224 also contribute to the local electrostatic potential, the effects of charges should be somewhat additive. To examine this possibility, we next investigated whether the effects of positive charges at sites 226, 228 and 260 are additive in the E224K mutant exhibiting strong intrinsic inward rectification (Yeh et al., 2005). Figure 4 shows that an additional H226E mutation alleviated partially the strong intrinsic inward rectification of the E224K mutant. The R260E mutation constructed in the E224K/H226E mutant further decreased the degree of intrinsic inward rectification. The E224K/H226E/R228E mutant did not result in functional expression. Replacing R260 with a neutral residue (A, G or Q) also decreased the degree of intrinsic inward rectification in the E224K/H226E mutant, but the effect was smaller compared to the R260E mutation. On the other hand, replacing R260 with a lysine did not affect the intrinsic inward rectification of the E224K/H226E mutant. The results shown in Figure 4 are consistent with the hypothesis that the charges at and near residue 224 contribute to the local electrostatic potential in

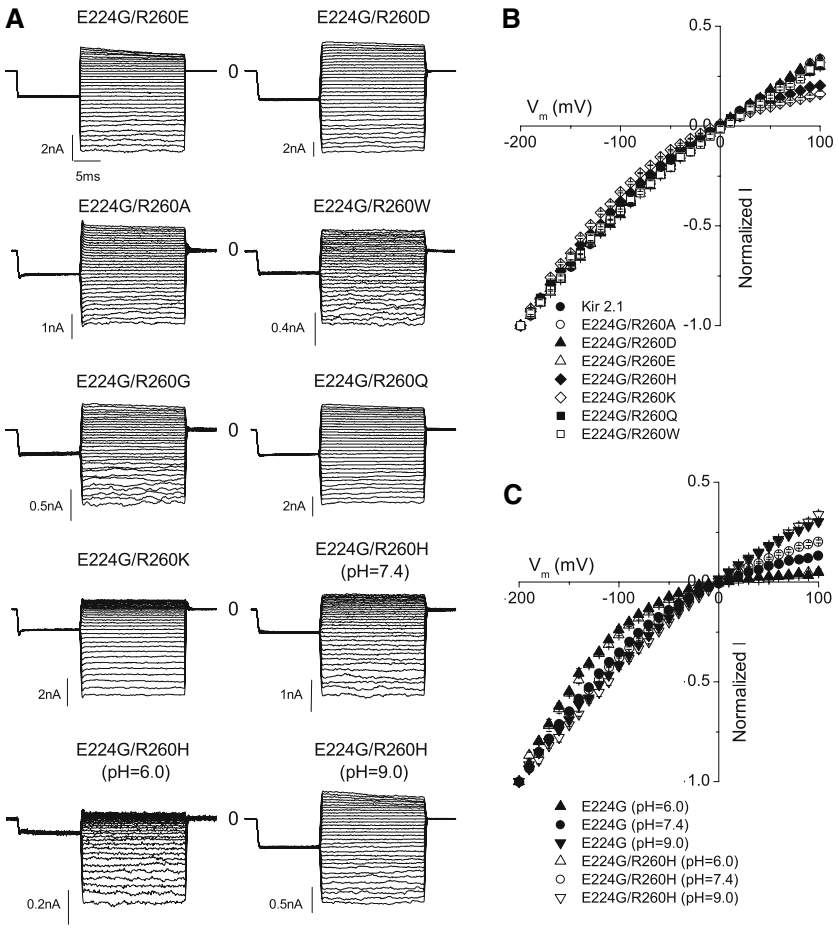

Fig. 3 Effects of size and charge of the side chain at residue 260 on the intrinsic inward rectification induced by E224G mutation of the Kir2.1 channel. (**a**) Macroscopic currents recorded from the indicated channel. (**b,c**) *I-V* relationships of the indicated channel. n = 3–9

the cytoplasmic pore and thus control intrinsic inward rectification.

Addition of Positive Charges at and Near Residue 223 in the Kir1.1 Channel Promoted Intrinsic Inward Rectification

We then examined whether the lack of inward rectification in the Kir1.1 channel (containing G223, which is equivalent to the E224G mutation of the Kir2.1 channel) was due to there being fewer positively charged residues near residue 223. Figure 5 shows that the wild-type channel conducted almost ohmically at V_ms ranging from –100 to +100 m*V*. However, the outward currents through the R-G223K and R-N259R mutants (R denotes mutation made in Kir1.1) were smaller than the inward currents at the same driving force, and the inwardly rectifying effect was even stronger in the R-G223K/N259R mutant. Furthermore, the R-G223E mutation was able to correct the inward rectification of the R-N259R mutant. No functional current could be detected in the R-Y227R mutant (more than 10 expression experiments using four different batches of oocytes). Thus, the effect of charges at residue 227 on the inward rectification and conductance of the Kir1.1 channel remains unknown.

In summary, we showed that the R-N259R, R-G223K and R-G223K/N259R mutants were more inwardly rectifying than the wild-type Kir1.1 channel in inside-out patches perfused with blocker-free solutions. The weaker inward rectification in the Kir1.1 channel than in the E224G mutant of the Kir2.1 channel is probably due to the fewer positively charged residues near site 223 compared to those of the E224G mutant.

Positively Charged Residues Near Residue 224 in the E224G Mutant Were Involved in the Open-Channel Fluctuations and Reduced Single-Channel Conductance

In addition to intrinsic inward rectification, the E224G mutant displays open-channel fluctuations. We next investigated how charge mutations at sites 226, 228 and 260 affect the single-channel conductance and kinetics of the E224G mutant. Figure 6a shows single-channel recordings from the indicated channel in inside-out patches held at –140 m*V*. The current through the wild-type Kir2.1 channel did not show open-channel fluctuations, whereas that through the E224G

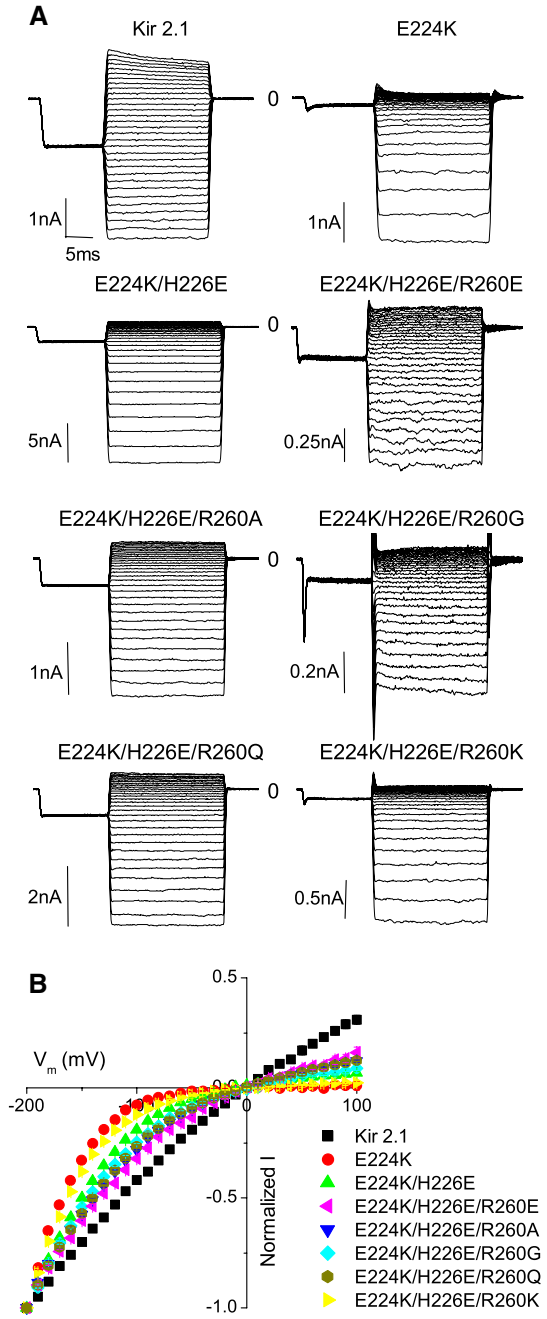

Fig. 4 The charge at residue 260 affected the intrinsic inward rectification of E224K/H226E mutants. (**a**) Macroscopic currents recorded from the indicated channel. (**b**) *I-V* relationships of the E224K, E224K/H226E and R260 mutations constructed in the E224K/H226E mutant. n = 3–6

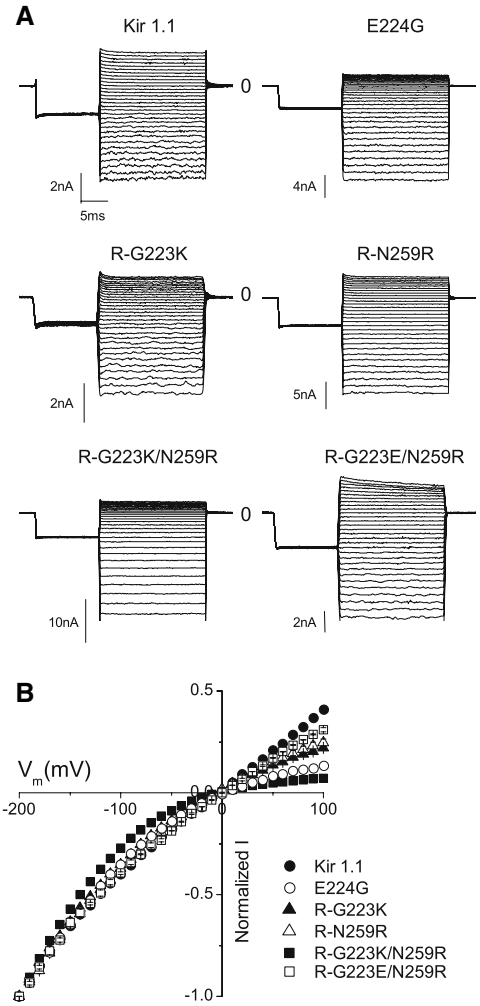

Fig. 5 Introduction of a positive charge at residue 223 or 259 made the Kir1.1 channel inwardly rectifying. (**a**) Macroscopic currents recorded from inside-out patches containing the indicated channel. (**b**) *I*-V relationship of the indicated channel. n = 3–6

mutant did. Replacing one of the positively charged residues 226, 228 or 260 in the E224G mutant with a glutamate completely eliminated the open-channel fluctuations and increased the single-channel current. Also, the E224G/R260D mutant did not show open-channel fluctuations and the single-channel current was larger than that in the wild-type channel. Neutralization of residue 260 also eliminated the open-channel fluctuations in the E224G mutant, and the single-channel currents were not significantly different from those for the wild-type channel. In contrast, the E224G/R260K and E224G/R260H mutants showed obvious open-channel fluctuations, although these were smaller than those in the E224G mutant. Furthermore, the single-channel current of the E224G/R260H mutant at pH_i 6.0 or 9.0 was, respectively, smaller or larger than at pH_i 7.4. Figure 6c shows the single-channel conductance of the wild-type Kir2.1 channel and mutants. Addition of a negative charge at residue 226, 228 or 260 in the E224G mutant increased the single-channel conductance; in addition, the single-channel conductance of these double-mutants was larger than in the wild-type Kir2.1 channel. Although neutralization of residue 260 also eliminated open-channel fluctuations, introduction of an extra negative charge at residue 260 resulted in increased single-channel conductance compared to the

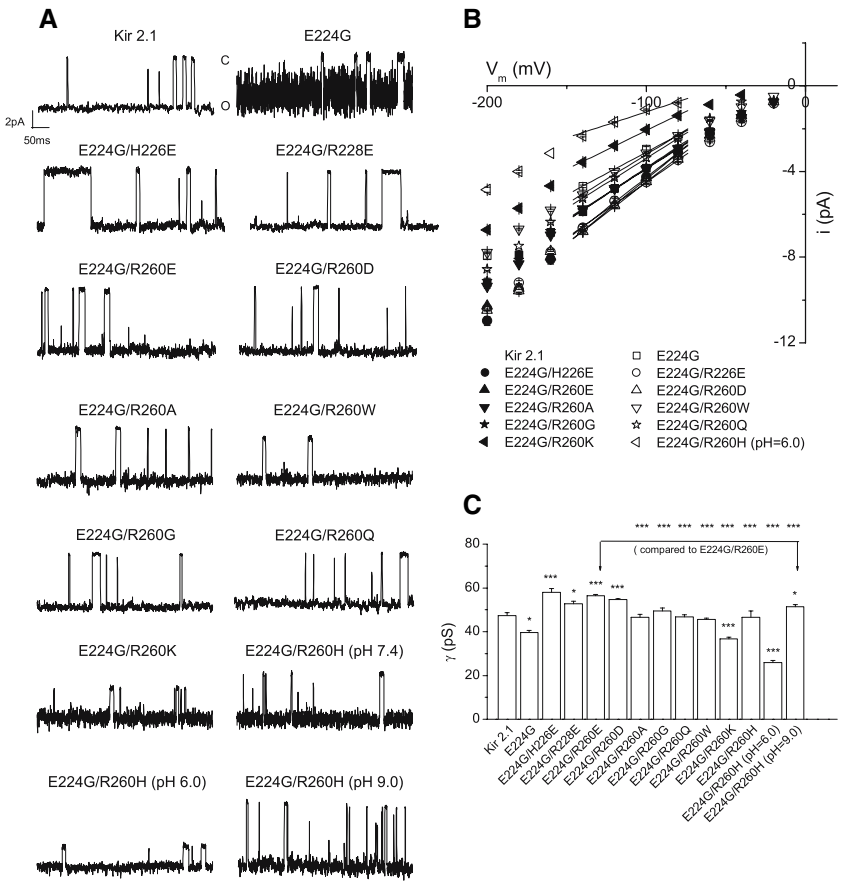

Fig. 6 Replacement of positively charged residues around residue 224 in the E224G mutant by a negatively charged or neutral residue eliminated the open-channel fluctuations and increased the single-channel conductance. (**a**) Single-channel traces of the indicated channel recorded at –140 m*V*. (**b**) *I-V* relationships of the indicated channel. (**c**) Single-channel conductance (γ) of the indicated channel. Single-channel conductance was measured from the slope of the *I-V* curve at V_ms ranging from –140 to –80 m*V*. n = 3–5; $^*p < 0.05$, $^{***}p < 0.005$

E224G/R260A, G, Q and W mutants. These results are consistent with the hypothesis that a negative electrostatic potential at and around residue 224 in the cytoplasmic pore is important in ensuring a smooth and large conductance of the Kir2.1 channel (Chang et al., 2005).

Note that extracellular divalent cations have been shown to reduce inward single-channel currents through Kir channels in native cells (Shioya, Matsuda & Noma, 1993) and cloned channels (Alagem, Dvir & Reuveny, 2001; Shieh, Chang & Arreola, 1998). We have also determined that the single-channel conductance is around 18 pS (slope conductance measured at V_m = –100 to –40 m*V*) in cell-attached patches exposed to an extracellular 100 mM $[K^+]$ and 3 mM free $[Mg^{2+}]$ in the Kir2.1 channel (Chang et al., 2005). In this study, single-channel currents were recorded in the absence of divalent cations in both extracellular and intracellular solutions and, thus, the single-channel conductance is larger than that reported in the presence of extracellular divalent cations.

Mechanism Underlying the Intrinsic Inward Rectification of the E224G Mutant

To understand the mechanism for the intrinsic inward rectification of the E224G mutant, we next examined both inward and outward single-channel currents through the E224G mutant. Figure 7a shows the single-channel traces of the E224G mutant at –100 and +100 m*V*, respectively. Both traces showed open-channel fluctuations. The lower panels of Figure 7a show the corresponding mean-variance histograms. In addition to the closed and main open states, an intermediate substate was observed at both –100 and +100 m*V*. The histograms shows that the open-channel fluctuations were mainly due to rapid transitions between the main open state and substate at –100 m*V* and between the closed state and substate at +100 m*V*. Figure 7b and c summarizes the kinetics and state probabilities of the E224G single-channel currents. The closed state exhibited two time components, which did not show strong V_m dependence. The probability of the channel entering the long closed state (C_L) was very low and V_m-independent but that of the short closed state (C_S) increased at positive V_m. The dwell time of the substate also showed little V_m dependence, and the probability of the channel in the substate was about the same at all values of V_m. On the other hand, both the dwell time and probability of the main state decreased when V_m became more depolarized. Furthermore, Figure 7d shows that the outward single-channel amplitudes of both the main open state and substate were smaller than the inward ones at the same driving force.

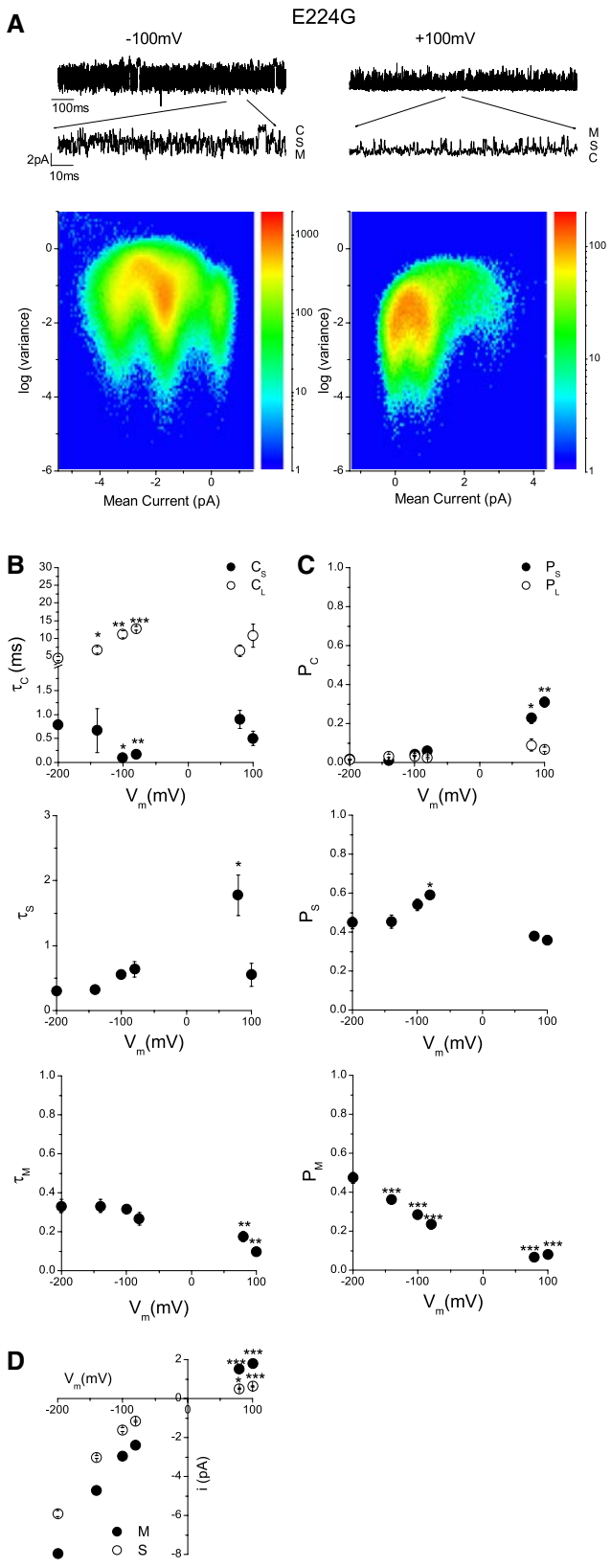

Fig. 7 V_m dependence of single-channel currents and kinetics in the E224G mutant. (**a**) Single-channel currents recorded in the E224G mutant at the indicated V_m. *I-V* histograms with a window of three points for the records. (**b**) V_m dependence of the dwell times at the closed state, substate and main open state. Dwell times at various V_m values were compared to those at –200 m*V*. (**c**) V_m dependence of the probabilities of the closed state, substate and main open state. Probabilities at various V_m values were compared to those at –200 m*V*. (**d**) *I-V* relationships for the substate and main open state. Current amplitudes at +80 and +100 m*V* were compared to those at –80 and -100 m*V*, respectively. n = 3–6; $*p < 0.05$, $**p < 0.01$, $***p < 0.005$

These results suggest that the intrinsic inward rectification of the E224G mutant is attributed to both V_m-dependent ion conductance and a V_m-dependent gating mechanism controlling the transitions between the closed state, substate and main open state.

The Cytoplasmic Pore of the E224G Mutant Reduced Single-Channel Conductance and Induced Open-Channel Fluctuations in the Kir1.1 Channel

Introducing positively charged residues at and around residue G223 in the Kir1.1 channel induced intrinsic inward rectification in the Kir1.1 channel (Fig. 5). Next, we examined the effects of these mutations on the single-channel conductance and open-channel fluctuations in the Kir1.1 channel. Figure 8a and b shows single-channel traces recorded at –140 m*V* and *I-V* relationships for the wild-type Kir1.1 channel, R-G223E, R-G223K, R-N259R, R-G223E/N259R and R-G223K/N259R mutants. The current amplitude of the R-G223E mutant was about the same as that of the wild-type Kir1.1, whereas those for the R-G223K and R-N259R mutants were smaller and that for the R-G223K/N259R mutant was even smaller still. Figure 8c shows that addition of a positive charge at residue 223 or 259 decreased the single-channel conductance to a similar extent and the R-G223K/N259R mutant reduced the single-channel conductance even more. The G223E mutation increased the single-channel conductance of the N259R mutant. These results show that the effect of charges at and near residue 223 in the Kir1.1 channel on the single-channel conductance was similar to that of charges near residue 224 in the Kir2.1 channel. Although the open-channel fluctuations appeared to be slightly larger in the R-G223K and R-N259R mutants compared to the wild-type Kir1.1 channel, they were much smaller than those in the E224G mutant of the Kir2.1 channel. It is possible that other factors present in the cytoplasmic pore of the E224G mutant but absent in that of the Kir1.1 channel are critical in inducing single-channel fluctuations.

To test this possibility, we constructed a chimera, $Kir1.1_{1–183}$-$Kir2.1_{185–428}$, by replacing the COOH terminus of the Kir1.1 (amino acids 184–391) with that of the Kir2.1 channel (residues 185–428, where site 185 is equivalent to site 184 in Kir1.1). A representative single-channel current of the $Kir1.1_{1–183}$-$Kir2.1_{185–428}$ chimera recorded at –140

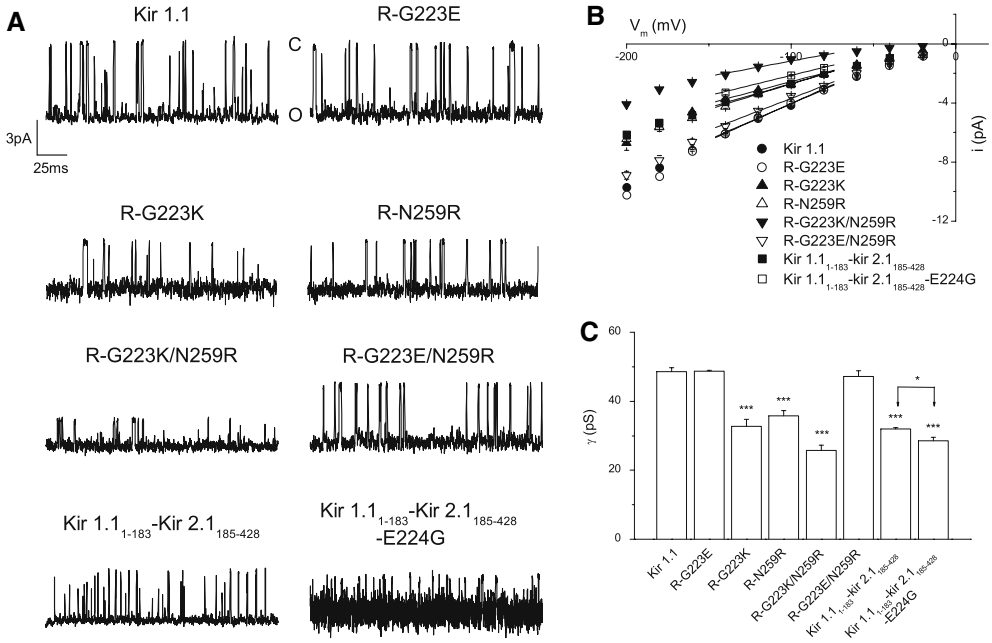

Fig. 8 Effects of charge mutations in the Kir1.1 channel and Kir1.1$_{1–183}$-Kir2.1$_{185–428}$ chimera on single-channel currents. (**a**) Single-channel traces of the indicated channel recorded at –140 m*V*. (**b**) *I-V* relationships of the indicated channel. (**c**) Single-channel conductance of the indicated channel. n = 3–6; $*p < 0.05$, $***p < 0.005$

m*V* is shown in Figure 8a. No open-channel fluctuations were observed in the chimera. The single-channel conductance of the chimera was smaller than that of the wild-type Kir1.1 channel (Fig. 8c). Similar results were previously observed (Choe, Sackin & Palmer, 2000; Taglialatela et al., 1994). It has been previously proposed that the single-channel conductance of the Kir1.2 (ROMK2) and Kir2.1 channels is the combined opposite contribution of the selectivity and cytoplasmic pores (Choe et al., 2000). While the conductance of the selectivity pore of the Kir2.1 channel is higher than that of the Kir1.2 channel, the cytoplasmic pore of the Kir2.1 channel is lower than that of the Kir1.2 channel (Choe et al., 2000). Therefore, the single-channel conductance of the Kir1.1$_{1–183}$-Kir2.1$_{185–428}$ chimera is smaller than that of the wild-type Kir1.1 channel.

We next constructed an additional E224G mutation in the Kir1.1$_{1–183}$-Kir2.1$_{185–428}$ chimera. Figure 8a shows that open-channel fluctuations were large in the Kir1.1$_{1–183}$-Kir2.1$_{185–428}$-E224G mutant and very similar to those in the E224G mutant of the Kir2.1 channel. Similar to the effect of the E224G mutant, the single-channel conductance of the Kir1.1$_{1–183}$-Kir2.1$_{185–428}$-E224G mutant was about 10% smaller compared to the Kir1.1$_{1–183}$-Kir2.1$_{185–428}$ chimera (Fig. 8c). These results are consistent with our hypothesis that the lack of a negative charge at site 223 in the Kir1.1 channel or at site 224 in Kir2.1 alone could not induce open-channel fluctuations in the Kir1.1/Kir2.1 channel. Other factors such as more positively charged residues near site 223/224 are also required to generate open-channel fluctuations. By introducing the cytoplasmic pore of the Kir2.1 channel into the Kir1.1 channel, open-channel fluctuations can be induced by additional E224G mutation.

E224G also induced intrinsic inward rectification in the Kir1.1$_{1–183}$-Kir2.1$_{185–428}$ chimera. Figure 9a shows mac-

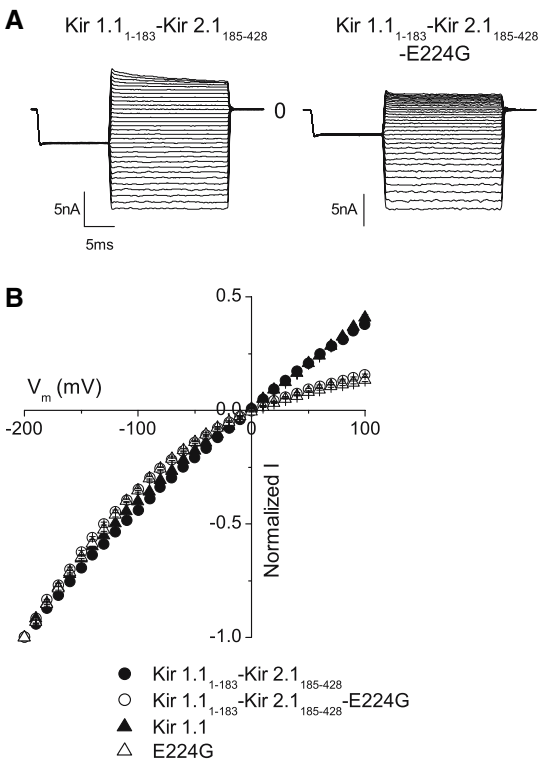

Fig. 9 E224G mutation induced intrinsic inward rectification in Kir1.1$_{1–183}$-Kir2.1$_{185–428}$ chimera. (**a**) Macroscopic currents recorded from inside-out patches containing the indicated chimera. (**b**) Normalized *I-V* relationships of the indicated channels. n = 4–5

roscopic currents through the Kir1.1$_{1–183}$-Kir2.1$_{185–428}$ and Kir1.1$_{1–183}$-Kir2.1$_{185–428}$-E224G chimeras exposed to Mg^{2+}- and polyamine-free solutions. The *I-V* relationship of the chimera with the E224G mutation showed a similar degree of intrinsic inward rectification to that of the E224G mutant of the Kir2.1 channel, whereas the *I-V* relationship of the Kir1.1$_{1–183}$-Kir2.1$_{185–428}$ chimera was almost linear and identical to that of the Kir1.1 channel (Fig. 9b).

Discussion

Positively Charged Residues in the Cytoplasmic Pore Are Involved in the Intrinsic Inward Rectification of the E224G Mutant

The E224G mutant of the Kir2.1 channel shows intrinsic inward rectification and open-channel fluctuations, whereas the Kir1.1 channel (in which the residue equivalent to E224 is a glycine) does not. This suggests that the lack of a negative charge at site 224 is not a necessary condition for the intrinsic inward rectification or open-channel fluctuations seen in the E224G mutant. In this study, we hypothesized that the positively charged residues near site 224 contribute to the local electrostatic potential in the cytoplasmic pore and thereby control the intrinsic inward rectification in the Kir2.1 channel. Our data showed that positively charged residues in the cytoplasmic pore were involved in the intrinsic inward rectification of the E224G mutant and that the presence of a greater number of positively charged residues around residue 224 in the E224G mutant than around G223 in Kir1.1 made the former more strongly rectifying and display larger open-channel fluctuations. A decrease in the number of positive charges at residues 226, 228 and 260 weakened the inward rectification induced by the E224G and E224K mutations, and the charge effects at residues 226 and 260 were additive. In addition, the introduction of positive charges at residues 223 and 259 or the replacement of the cytoplasmic pore of the Kir1.1 channel with that of the E224G mutant induced intrinsic inward rectification. These results suggest that positively charged residues at and near sites E224 and G223 are important factors determining the degree of intrinsic inward rectification in the Kir2.1 and Kir1.1 channels, respectively.

Outward currents through the wild-type channel declined with time at positive potentials (Fig. 2a). It has been reported that the time-dependent decay in the outward currents is due to the block of outward currents by intracellular HEPES and that replacing HEPES with KH_2PO_4 and K_2HPO_4 removes this effect (Guo & Lu, 2002). However, three groups, including our own, have shown that outward currents still decay using KH_2PO_4 and K_2HPO_4 (Fujiwara & Kubo, 2006; Xie, John & Weiss, 2002). Also, the time-dependent decay of outward currents through the Kir1.1$_{1–183}$-Kir2.1$_{185–428}$ chimera was more obvious than that of the Kir1.1 channel, suggesting that the decay is probably attributed to the cytoplasmic pore of Kir2.1 and is unlikely due to block of the channel by residual polyamines since N171 in the Kir1.1$_{1–183}$-Kir2.1$_{185–428}$ chimera should interact with polyamines with very low affinity. The mechanism for the time-dependent decay of the outward currents is unknown. It is possible that the decay of the outward currents is due to surface charge screening by the permeant K^+ ion in the cytoplasmic pore. In this study, we focused on comparing the instantaneous *I-V* relationships of the wild-type channels with those of the mutants.

The Kir1.2 channel and its N240R mutant, which is equivalent to the R-N259R mutant, do not show inward rectification in whole-cell recordings (Zhang et al., 2004). However, we showed here that the R-N259R mutant was more inwardly rectifying than the wild-type Kir1.1 channel. The reason for the discrepancy between the results obtained in the N259R mutant of the Kir1.1 channel and the N240R mutant of the Kir1.2 channel is unclear, but it may be due to the 19-amino acid difference between these two channels. The *I-V* relationship of the Kir1.1 channel shows weak inward rectification in whole-cell recordings (Ho et al., 1993), whereas that of the Kir1.2 channel is ohmic (Zhang et al., 2004). Thus, it is possible that the Kir1.1 channel is more sensitive than the Kir1.2 channel to the moderate increase in the electrostatic potential induced by the mutation.

In addition to charges at and near site 224, Pegan et al. (2005) proposes that a diaspartate cluster may act as a cytoplasmic regulatory element for inward rectification induced by polyamine block. However, it has been shown that a D259N mutation does not cause obvious intrinsic inward rectification in the Kir2.1 channel or affect the intrinsic inward rectification of the E224Q mutant (Fujiwara & Kubo, 2006). Whether D225 and D259 together contribute to local electrostatics by direct charge or a dipole effect and thus affect intrinsic inward rectification is unknown. Since in this study we focused on the difference between the Kir1.1 and Kir2.1 channels and the charges at these two sites are conserved at the equivalent sites in the two channels, these two residues were not examined.

Mechanisms Underlying the Intrinsic Inward Rectification of the E224G Mutant

Analysis of single-channel currents through the E224G mutant shows that the intrinsic inward rectification is due to the decreases of both single-channel conductance and

open probability at depolarized V_m. Many studies have shown that charges located in the wide pore mouths of ion channels can control ion conductance through electrostatic mechanisms (ion-concentrating and surface charge effects) (Brelidze, Niu & Magleby, 2003; Chandler, Hodgkin & Meves, 1965; Dani, 1986; Green, Weiss & Andersen, 1987; Green & Andersen, 1991; Hille, Woodhull & Shapiro, 1975; Imoto et al., 1988; Kell & DeFelice, 1988; Lin & Chen, 2003; MacKinnon, Latorre & Miller, 1989; Nimigean, Chappie & Miller, 2003; Xie et al., 2002). Consistent with the electrostatic hypothesis, we show in this study that the replacement of negatively charged residue at site 260 is more efficient than a neutral residue at decreasing the degree of intrinsic inward rectification in the E224K/H226E mutant and that the effect of H226E and R260E on the intrinsic inward rectification of the E224K mutant is additive. According to the structures of K^+ channels (Doyle et al., 1998; Kuo et al., 2003), the cytoplasmic pore is located outside the electrical field. A negative electrostatic potential in this region will be important in accumulating K^+ ions from the internal side and subsequently facilitating K^+ entry into the water cavity upon depolarization. An increase in local potential (lower negative potential) by mutagenesis or by binding of cationic polyamines to this region will not only decrease the K^+ gradient into the water cavity but also increase the energy barrier for K^+ flowing into the water cavity and thus decrease outward currents. Since the inward K^+ flux is affected more by the ion-ion interaction in the selectivity filter than by the electrostatic potential in the cytoplasmic pore and since the electrochemical gradient favors K^+ influx during hyperpolarization, the electrostatic potential affects K^+ efflux less than K^+ influx. As a result, inward rectification becomes stronger when the electrostatic potential in the cytoplasmic pore is increased. We have tried to estimate the electrostatic potential near site 224 of the wild-type Kir2.1 channel by measuring the effects of charge mutations on the rate constants for the reactions of sodium (2-sulfonatoethyl)-methanethiosulfonate (MTSES) and [2-(trimethylammonium) ethyl]-methanethiosulfonate (MTSET) with cysteine mutants in the cytoplasmic pore as previously described (Lin & Chen, 2003; Stauffer & Karlin, 1994). However, the rates for MTSES modification were too small ($< 10\ M^{-1}s^{-1}$) to allow accurate estimation of local electrostatic potential.

The H226E and H226E/R260E mutations did not completely convert the *I-V* relationship of the E224K mutant to that of the wild-type channel, although the net negative charge of the former mutant was equal to, and that of the latter greater than, that of the wild-type channel (Fig. 4). These results indicate that other factors, such as the side chains of residues, the geometrical distribution of charges and local conformational changes, may also affect the intrinsic inward rectification.

Positively Charged Residues in the Cytoplasmic Pore Decrease the Single-Channel Conductance and Are Involved in Generating Open-Channel Fluctuations

In addition to intrinsic inward rectification, the positive charges near residue 224 in the cytoplasmic pore of the Kir2.1 channel determine the conductance and are responsible for the occurrence of open-channel fluctuations observed in single-channel recordings. Our results showed that the open-channel fluctuations induced by the E224G mutation were completely removed when residue 260 was neutralized or when one of the positively charged residues at 226, 228 and 260 was replaced with a negatively charged residue. Neutralization of residue 260 increased the single-channel conductance of the E224G mutant to the level seen with the wild-type Kir2.1 channel. Moreover, the single-channel conductance of the E224G/H226E, E224G/R228E, E224G/R260E or E224G/R260D mutant was larger than that in the wild type. These results show that charge effects on single-channel conductance are additive. We have shown that the E224G mutation generates a substate and that the open-channel fluctuations are due to rapid transitions between the closed state, substate and main open state (Fig. 7) (Chang et al., 2005). Previous studies have also shown that the charge effect in the cytoplasmic pore of the E224G mutant on open-channel fluctuations is probably due to an electrostatic mechanism (Chang et al., 2005; Fujiwara & Kubo, 2006; Xie et al., 2004). However, the underlying mechanisms for the intrinsic inward rectification and for the rapid transition between different states may be different. For example, the R-G223K and R-N259R mutants show intrinsic inward rectification but fail to induce open-channel fluctuations. Similarly, the interaction of spermine with E224 and E299 produces intrinsic inward rectification without blocking the Kir2.1 channel pore (Yeh et al., 2005). However, spermine does not cause open-channel fluctuations in the Kir2.1 channel (Xie et al., 2002). Furthermore, the open-channel fluctuations in the E224G, E224G/R260K and E224G/R260H mutants are quantitatively different. These results suggest that open-channel fluctuations may require a mechanism in addition to the long-range electrostatic effect.

In addition to electrostatic effects, charged residues may contribute to ion permeation through close interaction with permeant ions. The outer ring of negatively charged residues within the extracellular pore of a nicotinic acetylcholine receptor has been previously proposed to concentrate ions within the pore through long-range electrostatic interaction and thus to contribute to net ion conductivity (Imoto et al., 1988). However, detailed examination of charge changes in the outer ring (Kienker et al., 1994) and direct electrostatic potential measurement using lanthanide-based diffusion-enhanced fluorescence

energy transfer experiments (Meltzer et al., 2006) suggest that the effect of the negatively charged ring is predominantly through close interactions with permeant ions instead of long-range electrostatic interaction. It is possible that residues at and near site 224 may facilitate ion permeation through close interaction with K^+. Our previous study (Chang et al., 2005) with various permeant ions suggests that occupancy of the K^+ ion in the cytoplasmic pore may gate the Kir2.1 channel in an electrostatically dependent way. When the electrostatic potential increased, K^+ occupancy in the cytoplasmic pore occurred less frequently and the channel therefore fluctuated between a high conductance state (main open state, with K^+ occupancy in the cytoplasmic pore) and a low conductance state (substate, no K^+ occupancy). Furthermore, it has been previously shown that bigger neutral side chains at site 224 exert larger effects on open-channel fluctuations and the degree of intrinsic inward rectification in the Kir2.1 channel (Xie et al., 2004). This result suggests that in addition to the electrostatic effect, the side-chain size of the substituted residue at and near site 224 has a steric effect on K^+ permeating through the cytoplasmic pore.

It has recently been shown that the R228Q mutation eliminates the open-channel noise induced by the E224Q mutation in the Kir2.1 channel and increases the single-channel conductance (Fujiwara & Kubo, 2006). However, the single-channel conductance of the E224Q/R228Q mutant is still smaller than that of the wild-type channel. Also, the degree of inward rectification in the E224Q mutant is decreased by an additional R228Q mutation, but it is still larger than that of the wild-type channel (Fujiwara & Kubo, 2006). Three possibilities may explain the different effects of charge neutralization of residue 228, seen in the above study, and of residue 260, seen in our present study. First, the additional mutation was constructed at different sites (residue 228 *vs.* 260). Second, the additional mutation was generated on a different mutation background (E224G *vs.* E224Q). Third, the single-channel recordings were measured in different patch configurations (cell-attached *vs.* inside-out recordings [in which the effect of surface charge screening by polyamines in not seen]). We also showed that single-channel conductance was reduced and that open-channel fluctuations were smaller in the E224G/R260K mutant and in the E224G/R260H mutant at pH_i 6.0 than in the E224G mutant. The open-channel fluctuations are due to rapid transitions between a main open state and a substate (Chang et al., 2005). It is possible that the levels of both states are reduced in the E224G/R260K and E224G/R260H mutants to such a degree that the difference in the conducting levels is small. Thus, the fluctuations would be smaller in the E224G/R260K and E224G/R260H (pH_i 6.0) mutants than in the E224G mutant.

In conclusion, in addition to negative charges, positive charges in the cytoplasmic pore control the intrinsic inward rectification and conductance of Kir1.1 and Kir2.1 channels, possibly by contributing to the local electrostatic potential. When the electrostatic potential is more negative (e.g., in the absence of polyamines), conductance is high and inward rectification, weak. As the electrostatic potential is increased (e.g., in the presence of polyamines or by addition of positive charges), inward rectification is strong, conductance is reduced and the substate occurs frequently.

Acknowledgement We thank Drs. Lily Jan and James N. Weiss for kindly providing the Kir2.1 and Kir1.1 clones, respectively. We are grateful to Dr. Tom Barkas for reading and editing the manuscript. This work was supported by the Academia Sinica and by the National Science Council of Taiwan (grants 94–2320-B-001–025 and 95–2320-B001–011).

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