K⁺ Binding Sites and Interactions between Permeating K⁺ Ions at the External Pore Mouth of an Inward Rectifier K⁺ Channel (Kir2.1)*

Ru-Chi Shieh‡, Jui-Chu Chang‡, and Chung-Chin Kuo¶

From the ‡Institute of Biomedical Sciences, Academia Sinica, Taipei 11529 and the ¶Department of Physiology, National Taiwan University College of Medicine and Department of Neurology, National Taiwan University Hospital, Taipei 101, Taiwan, Republic of China

The arginine at position 148 is highly conserved in the inward rectifier K⁺ channel family. Increases of external pH decrease the single-channel conductance in mutant R148H of the Kir2.1 channel (arginine is mutated into histidine) but not in the wild type channel. Moreover, in 100 mM external K⁺, varying external pH induced biphasic changes of open channel noise, which peaks around pH 7.4 in the R148H mutant but not in the wild type channel. The maximum single-channel conductances are higher in the wild type channel and R148H mutant at pH 6.0 than those in the R148H mutant at pH 7.4. However, the maximal conductance is achieved with much lower external [K⁺] for the latter. Interestingly, the single-channel conductances and open channel noise of the wild type channel at pH 6.0 and the R148H mutant at pH 6.0 and 7.4 become the same in [K⁺] = 10 mM. These results indicate that the residue at position 148 is accessible to the external H⁺ and probably is involved in the formation of two K⁺ binding sites in the external pore mouth. Effective repulsion between permeating K⁺ ions in this area requires a positive charge at position 148, and such K⁺-K⁺ interaction is the essential mechanism underlying high K⁺ conduction rate through the Kir2.1 channel pore.

Inward rectifier K⁺ channels are important in maintaining stable resting membrane potentials and controlling excitability of many cells. The physiological function of these channels is closely related to their unique inward rectification mechanisms, which allow much larger inward current than outward current through the channels. Another important “asymmetrical” feature of the inward rectifier K⁺ channels is that external but not internal K⁺ seems to interact with and thereby activate the channel (1). The mechanisms underlying inward rectification have been ascribed to voltage (V_m)-dependent blockade by internal cations such as Mg²⁺ (2, 3) and polyamines (4, 5) and to a pH-dependent intrinsic gate (6). On the other hand, the ion permeating process itself, namely interactions between K⁺ ions and between the permeating K⁺ ion and the inward rectifier K⁺ channel, is less characterized.

The arginine at position 148 is highly conserved in the inward rectifier K⁺ channel family and seems to play an important role in the K⁺ ion permeation process. Kubo (7) showed that mutation of the arginine at position 148 into tyrosine (R148Y) in the cloned inward rectifier K⁺ channel (Kir2.1) (8) resulted in a reduction of the [K⁺]₀-dependent activation of inward currents and a negative shift of the conductance-voltage relationship. Sabirov et al. (9) reported that although mutation of the arginine at position 148 into histidine (R148H) resulted in no channel activity, coexpression of the mutant with the wild type (WT) cRNA demonstrated populations of channels with reduced single-channel conductances (γ). Moreover, the single-channel conductance is dramatically increased in a recently cloned inward rectifier K⁺ channel (Kir7.1) when the neutral amino acid methionine at position 125 (the equivalent position of the arginine at site 148 in Kir2.1) was mutated into a positively charged amino acid (10). All these studies suggest that the positive charge at position 148 (148R in Kir2.1) when the neutral amino acid methionine at position 125 (the equivalent position of the arginine at site 148 in Kir2.1) was mutated into a positively charged amino acid (10). All these studies suggest that the positive charge at position 148 (148R in Kir2.1) when the neutral amino acid methionine at position 125 (the equivalent position of the arginine at site 148 in Kir2.1) was mutated into a positively charged amino acid (10).

With successful expression of the Kir2.1 mutant R148H in Xenopus oocytes, we studied the effect of pH, and [K⁺], on the single-channel conductance and the open channel “noise” in WT channel and R148H mutant. We found that the side chain of the amino acid at position 148 is accessible to the external H⁺. Also, the amino acid at position 148 probably is involved in the formation of a set of two K⁺ binding sites in the external pore mouth. Effective repulsion between permeating K⁺ ions in this area requires a positive charge at position 148, and such K⁺-K⁺ interaction may be an important mechanism underlying high K⁺ conduction rate through the Kir2.1 channel pore.

**EXPERIMENTAL PROCEDURES**

Molecular Biology and Preparation of Xenopus Oocytes—Mouse macrophage Kir2.1 in pCDNA1/Amp (generously provided by Dr. Lily Jan (University of California at San Diego)‡) digested with HindIII and SmaI was subcloned into the HindIII-SmaI sites in pALTEKTet (Promega, Madison, WI, ‡). Site-directed mutation was then generated using the Altered Sites II in vitro mutagenesis systems (Promega, Madison, WI). The mutant DNA was sequenced with the ABI Prism™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Bio

1 The abbreviations used are: WT, wild type; 148R, the positive charge at position 148; NMG, N-methyl-D-glucamine; MES, 2-morpholinoethanesulfonic acid; CHES, 2-cyclohexylaminoethanesulfonic acid; pS, picosiemens.
systems, Foster City, CA) to confirm the presence of histidine at position 148. The WT Kir2.1 subcloned in pBSIISK(1) (a generous gift from Drs. Scott A. John and James N. Weiss) and R148H DNAs were linearized with NotI and ScaI, respectively, and approximately 1 mg of purified linear DNA was used for in vitro T7 (for WT DNA) and Sp6 (for R148H DNA) transcription reactions (mMessage mMachine, Ambion, Dallas, TX).

Xenopus oocytes were prepared as described previously (11). In brief, Xenopus oocytes were isolated by partial ovariectomy from frogs anesthetized with 0.1% aminobenzoic acid ethyl ester. The day after isolation, Xenopus oocytes were pressure-injected with either 10–100 pg of WT cRNA or 100–1000 pg of R148H cRNA. Oocytes were maintained at 18 °C in Barth’s solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.3 mM Ca(N₂O₆), 0.41 mM CaCl₂, 0.82 mM MgSO₄, 15 mM HEPES, 20 μg/ml gentamicin, pH 7.6, and used 1–3 days after RNA injection.

Single-channel Recordings—Single-channel currents were recorded at room temperature (22–24 °C) on cell-attached or inside-out patches (12) using an Axopatch 200A amplifier (Axon Instruments, Burlingame, CA). Glass electrodes were pulled using a horizontal electrode puller...
(Sutter, Novato, CA) from borosilicate glass tubing (WPI Inc., Saratoga, FL). The diameters of the pipettes ranged from 1 to 5 μm. The command voltage pulses and data acquisition functions were processed using a Pentium 100 computer, a DigiData board, and pClamp6 software (Axon Instruments, Burlingame, CA). Data were sampled at 5–10 kHz and filtered at 1–5 kHz.

For experiments performed in cell-attached patches, the pipette solution contained 100 mM KCl + KOH, 2 mM EDTA. For experiments carried out in inside-out patches (except for Fig. 7; see Fig. 7 legend), the pipette and internal solutions were always kept symmetrical and both contained x mM KCl + KOH, 2 mM EDTA, where x = 10–1000. For x = 10–100, 90 mM of N-methyl-D-glucamine (NMG) was also added.

Because the single-channel conductances obtained with 100 mM [K+] and 100 mM [K+] + 90 mM [NMG] are similar, NMG was not added for solutions containing [K+] > 100 mM. Because it has been shown that channels formed from coexpression of the WT and R148H subunits are sensitive to external divalent cations (9), in this study we used pipette solutions containing no divalent cations. The patch electrode solution was pH-buffered by the following buffers: 5 mM MES for pH  < 7.0; 5 mM HEPES for 7.0 ≤ pH ≤ 8.0; 5 mM CHES for pH > 8.0. The rundown of channel activity was avoided by treating the inside-out patches with 25 μM of 1,2-dioleoyl-sn-glycero-3-phosphatidylinositol-4,5-bisphosphate (S igna ma) (11, 13).

**Data Analysis**—Single-channel current amplitude was measured by manually placing a cursor at the center of the closed or the open state levels and from amplitude histograms. To compare the degree of open channel noise in the WT channel and R148H mutant under various recording conditions, current variance (σ) of single-channel recordings was calculated for the open and closed states using the Clampfit basic statistics:

\[
\sigma = \frac{1}{N-1} \sum_{i=1}^{N} (x_i - \bar{x})^2
\]

where \( \bar{x} \) is the mean of the samples, and N is the sample number. \( \bar{x} \) was calculated from a segment in the open or closed level current of the sample sweeps (187–506 sample points). The open channel current variance from a sweep was normalized to the closed level current variance of the same sweep, and the normalized σ(O)/σ(C) values were then used to compare the magnitude of open channel noise among different experimental conditions. Averaged data are presented as the means ± S.E. Student’s independent t test was used to assess statistical significance.

**Fig. 3. Effects of pH on the i-Vm relationships of the R148H mutant.** Single-channel currents were measured in inside-out patches (n = 2–5) exposed to symmetrical 100 mM [K+] at pH5 = 7.4 and the indicated pH5.

**Fig. 4. The Vm dependence of the effect of pH5 on single-channel currents.** A, the fractional currents (averaged single-channel currents recorded at various pH5 normalized to that at pH5 = 6.0), iVm/σ(O), are plotted against pH5. The curves are the best fits to data of the form: \( [H^+] / [H^+] + K_0 \) + C where C = 0.15 (--140 mV), 0.08 (--120 mV), 0.11 (--100 mV), 0.09 (--80 mV), or 0.08 (--60 mV). B, Ks values obtained from panel A are very similar at Vm = –60 to –140 mV.

**Fig. 5. Effects of pH on open channel noise.** Single-channel currents were recorded (with a sampling rate of 10 kHz and filtering rate of 1 kHz) in cell-attached patches exposed to 100 mM [K+] at different pH5. Open channel noises were then quantified and expressed as σ(O)/σ(C) (see “Experimental Procedures”) for the WT channel (A) and R148H mutant (B). Data groups shown in panel A are not statistically different from each other (p > 0.05), whereas the particular data group indicated by an asterisk in panel B is statistically different from that of the R148H mutant at pH5 = 7.4 (p < 0.001; n = 18 in each data group).
RESULTS

Expression of the R148H Mutant in Xenopus Oocytes—Fig. 1A shows a series of sample current traces recorded at testing pulses in a cell-attached patch containing one single R148H mutant channel at pH 7.0. Fig. 1B shows the single-channel current amplitude histogram demonstrating a single predominant conducting state. The averaged single-channel current-voltage ($i$-$V_m$) relationship is plotted in Fig. 1C. The $i$-$V_m$ relationship of the R148H mutant remains inwardly rectifying, and no outward single-channel current is recorded.

Monotonously Decreased Single-channel Conductance of the R148H Mutant with Increasing $pH_o$—After successful expression of the R148H mutant in Xenopus oocytes, we explored the role of 148$^+$ in $K^+$ permeation. First we examined whether the histidine residue is accessible to external $H^+$ and thus the charge at position 148 can be controlled by varying $pH_o$. Fig. 2A shows the single-channel currents recorded at $V_m = -140$ mV from a holding potential of 0 mV at various $pH_o$ in the WT channel and R148H mutant. Changes of $pH_o$ have little effect on the single-channel current in the WT channel, yet the single-channel current amplitude becomes smaller at higher (more alkaline) $pH_o$ in the R148H mutant. At low $pH_o$, the single-channel current in the mutant is almost comparable with (although still slightly smaller than) the current in the WT channel. As $pH_o$ is increased, the single-channel current of the R148H mutant decreases in a $pH_o$-dependent manner. The averaged single-channel currents recorded from several patches similar to that shown in Fig. 2A are plotted against membrane potentials and are shown in Fig. 2B. The $i$-$V_m$ relationships are very similar at $pH_o$ 5.0 to 6.5. Further increase of $pH_o$ decreases single-channel conductance in a $pH_o$-dependent manner. Fig. 2C shows that the conductance of the WT channel is little affected by changes in $pH_o$, which is consistent with previous studies (9, 14). In contrast, the single-channel conductance of the R148H mutant is sensitive to $pH_o$. The $\gamma$-$pH_o$ relationship can be described by a simple one-to-one binding curve with a $K_d$ of 7.3, and $\gamma$ varies between 26 and 7 pS. These findings strongly support that the side chain of histidine at position 148 can be protonated by external $H^+$, and 148$^+$ seems to enhance $K^+$ ion permeation through the Kir2.1 channel pore.

$pH_i$ Has No Effect on the Single-channel Conductance of R148H Mutant—The effect of $pH_i$ on the R148H mutant was also examined. Fig. 3 shows that changes of $pH_i$ from 6.0 to 9.0 do not produce any change of the single-channel conductance of the R148H mutant. Thus the histidine at position 148 seems to
be accessible only to external H\(^+\) but not to internal H\(^+\).

Lack of \(V_m\) Dependence of the p\(K_a\) of R148H Mutant—To determine whether the amino acid at position 148 is located within the electrical field, \(V_m\) dependence of the effects of pH on the single-channel current of the R148H mutant was examined. At each \(V_m\), the averaged single-channel currents recorded at different pH were normalized to those at pH 6.0 (Fig. 4A). The normalized \(i\)-\(pH\) relationships are similar at different \(V_m\). Fig. 4B shows that the p\(K_a\) values determined from the \(i\)-\(pH\) relationships in Fig. 4A remain very similar at different \(V_m\). The lack of \(V_m\) dependence of p\(K_a\) suggests that the amino acid at position 148 is probably located outside the electrical field.

Biphasic Changes of Open Channel Noise with pH\(_o\)—It is interesting to note that other than single-channel conductances, the open channel noises also vary with pH\(_o\) in the R148H mutant. For example, it is evident in Fig. 2A that the open channel noise increases when pH\(_o\) is increased from 5.0 to 7.4. However, further increases of pH\(_o\) result in a decrease in open channel noise. To compare open channel noise we calculated single-channel current variance at the open channel level and then normalized it to that at the closed level in the same sweeps (for details see under “Experimental Procedures”). Fig. 5 demonstrates the normalized variance of single-channel currents in the WT channel (Fig. 5A) and in the R148H mutant (Fig. 5B) at different pH\(_o\). The open channel noise varies with pH\(_o\) in a biphasic manner and is peaked at pH\(_o\) 7.4 in the R148H mutant, whereas the open channel noise remains small and does not change with pH\(_o\) in the WT channel. The biphasic change of the open channel noise in the R148H mutant seems to indicate that the unitary conductance of the mutant channel quickly fluctuates between two levels. The two levels probably are correlated with protonation and deprotonation of residue 148 of the channel, because the fluctuation is smallest either at pH\(_o\) 6.0 (where the channel is mostly in the protonated and high conductance state) or at pH\(_o\) 9.0 (where the channel is mostly in the deprotonated and low conductance state) yet is most prominent at pH\(_o\) 7.4, the p\(K_a\) of the histidine residue at position 148 in the R148H mutant (Fig. 4).

Higher Affinity between Permeating K\(^+\) Ions and the R148H Mutant Channel—At pH\(_o\) 7.4 but not at pH\(_o\) 6.0—We have demonstrated that histidine at position 148 of the R148H mutant can be accessed by H\(^+\) from the external space and that protonation of this site may have a significant effect on ion permeation through the Kir2.1 channel. The biphasic effect of pH\(_o\) on the open channel noise furthermore argues for a direct rather than an indirect or allosteric effect of 148\(\ominus\) on K\(^+\) permeation through the Kir2.1 channel (see “Discussion” for details). A direct effect of a positive charge (148\(\oplus\)) on the permeating K\(^+\), a positively charged ion, most likely would be an elevation in the free energy experienced by K\(^+\). Because the single-channel conductance is higher in channel with 148\(\ominus\), what is likely to be elevated is the minimum rather than the maximum of the energy profile. Because an increase in energy minimum may be manifested as a decrease in binding affinity for K\(^+\) in the pore, we investigated the effects of varying [K\(^+\)] on the single-channel conductance of the WT channel and R148H mutant. The \(i\)-\(V_m\) relationships were measured in symmetrical [K\(^+\)] ranging from 10 to 1000 mM in inside-out patches exposed to internal Mg\(^{2+}\)- and polyamine-free solutions. Fig. 6 shows the \(i\)-\(V_m\) curves for the WT channel at pH\(_o\) 6.0, R148H mutant at pH\(_o\) 6.0, and R148H mutant at pH\(_o\) 7.4 (panels A–C, respectively). Fig. 6D plots the single-channel conductance against [K\(^+\)] (\(\gamma\)-[K\(^+\)] plot) for the WT channel at pH\(_o\) 6.0, R148H mutant at pH\(_o\) 6.0, and R148H mutant at pH\(_o\) 7.4. For the WT channel at pH\(_o\) 6.0, the single-channel conductance steeply increases as [K\(^+\)] is increased and reaches a plateau of ~53 pS with a \(K_d\) = 49 mM. The [K\(^+\)] dependence of the single-channel conductance of the R148H mutant at pH\(_o\) 6.0 is similar to the WT channel except that the single-channel conductance saturates at 35 pS with a \(K_d\) = 30 mM. In contrast, the single-channel conductance of the R148H mutant at pH\(_o\) 7.4 saturates at ~14 pS. Because the single-channel conductance is already ~10 pS in 10 mM [K\(^+\)], it is plausible that the \(K_d\) for K\(^+\) binding in the R148H mutant is smaller than 10 mM (an accurate estimate of \(K_d\) is difficult in this condition because it is hard to accurately measure the single-channel conductance with [K\(^+\)] < 10 mM). Thus the affinity between K\(^+\) and the R148H mutant channel pore seems to be higher at pH\(_o\) 7.4 than at pH\(_o\) 6.0. This is consistent with the foregoing prediction that 148\(\ominus\) elevates the energy minimum experienced by the permeating K\(^+\) in the pore.

It is also interesting to note in Fig. 6D that the single-channel conductances for the three experimental conditions are the same in [K\(^+\)] = 10 mM. If the increased open channel noise in 100 mM [K\(^+\)] (Fig. 5) is indeed ascribed to the fluctuations of the single-channel conductance between two conducting levels and thus results in the decreased conductance of the R148H mutant in 100 mM [K\(^+\)] at pH\(_o\) 7.4, it would be desirable to check whether the open channel noises would also be the same in 10 mM [K\(^+\)] in different experimental conditions, just like the single-channel conductance. Fig. 6E shows this is indeed the case. The open channel noises are about the same for the WT channel at pH\(_o\) 6.0, R148H mutant at pH\(_o\) 6.0 and R148H mutant at pH\(_o\) 7.4 in 10 mM [K\(^+\)]. Moreover, the open channel noises in these conditions are all small and similar to the noises in the WT channel and R148H mutant at pH\(_o\) 6.0 in 100 mM [K\(^+\)]. Thus the effect of protonation at position 148 in the R148H mutant, or 148\(\ominus\), on K\(^+\) conductance seems to be different in different bulk K\(^+\) concentration, suggesting that the free energy profile of the permeating K\(^+\) ion in the pore actually varies with bulk K\(^+\) concentration.

Internal [K\(^+\)] Has No Effect on the pH\(_o\)-dependent Changes of
the Single-channel Conductance in the R148H Mutant—We have demonstrated that 148$^+$ has a significant effect on ion permeation through Kir2.1 channel pore, and the effect is closely correlated with bulk K$^+$ concentration. Because the results in Fig. 6 are obtained in symmetrical [K$^+$] and the results in Figs. 1–4 suggest that the residue at position 148 is located in the external pore mouth, it is desirable to further examine whether it is external K$^+$ or internal K$^+$ that is crucial for the effect of 148$^+$ on K$^+$ permeation. Fig. 7 shows the effects of asymmetrically varying [K$^+$] on the single-channel conductance of the R148H mutant at pH 7.4. In these experiments, the pipette solution contained either 10 or 100 mM [K$^+$], and the inward currents were recorded at various $V_m$ in inside-out patches perfused with either 10 mM [K$^+$] or 100 mM [K$^+$]. The results indicate that the single-channel conductance of the R148H mutant is dependent on [K$^+$], but not internal [K$^+$] ([K$^+$]). The $V_m$ dependence of the effect of pH (Fig. 4) and the lack of effect of pH (Fig. 3) as well as [K$^+$] (Fig. 7) on the single-channel conductance of the R148H mutant all suggest that position 148 is located very close to the external pore mouth.

DISCUSSION

In this study, we report successful expression of the Kir2.1 mutant R148H in *Xenopus* oocytes. We find that to obtain the same level of functional channel expression, the amounts of the R148H cRNA injected into the *Xenopus* oocytes have to be about 10–100 times more than those of the WT cRNA. The fact that the R148H mutant retains inward rectification (because of V$m_\text{a}$-dependent block of the channels by intracellular Mg$^{2+}$ and polyamines), sensitivity to extracellular Ba$^{2+}$ block (data not shown), and K$^+$ selectivity (9) suggests the absence of major conformational changes caused by the mutation. Our results show that the side chain of the amino acid residue at position 148 is accessible to external H$^+$ and that this amino acid is probably located outside the electrical field. Furthermore, protonation of this position is essential for a faster transportation of permeating K$^+$ ions. Because changes of pH from 5.0 to 9.0 do not have any significant effect on the single-channel conductance of the WT channel (Fig. 2), external H$^+$ (or OH$^-$) binding to a part of the channel other than at position 148 probably can be neglected as a first approximation in the discussion of the findings here. Basically there are two possible mechanisms underlying the effect of pH at position 148: protonation-deprotonation at this position may have an indirect or allosteric effect on channel protein and thus on K$^+$ permeation, or it may have a direct effect on K$^+$ permeation through the Kir2.1 channel pore.

If pH$_i$, modulated K$^+$ permeation through the pore by an allosteric effect, the single-channel conductance in the R148H mutant at pH$_o$ 6.0 presumably should have a more constant relationship with that at pH$_o$ 7.4 in any given [K$^+$]. It is therefore difficult to envisage why the single-channel conductances would be the same for the R148H mutant at pH$_o$ 6.0 and 7.4 in 10 mM [K$^+$] yet are quite different in 100 mM or higher [K$^+$] (Fig. 6D). Moreover, in Figs. 2A and 5 we have noted that pH$_i$ has an effect on both the open channel noise and the single-channel conductance. Most interestingly, the open channel noise has a biphasic change. When pH$_i$ is increased from 6.0 to 7.4 the open channel noise increases. However, further increasing pH$_i$ results in a decrease in the open channel noise. With an allosteric model, it is hard to envisage the biphasic effect on the open channel noise by pH$_i$. Also, with a sampling interval of 100 μs, we still could not resolve the open channel noise into discrete conducting states, indicating that the effect of protonating the channel has very fast kinetics. Because protein conformational change usually is a much slower proc-
ess, it seems unlikely that an allosteric effect involving large scale protein conformational changes is responsible for the pHo effect in the R148H mutant.

In contrast to the indirect or allosteric model, a direct effect of external H+ on K+ ion conduction seems much more plausible. A direct effect of protonation-deprotonation in the pore will certainly fit the very fast kinetics of the modulatory effect of external H+ (revealed by the increased open channel noise).

In view of higher single-channel conductance in channels with 148Δ (Fig. 2), we have argued that the direct effect of 148Δ may involve elevation of some energy minimum rather than energy maximum of the permeating K+ ion. The findings in Fig. 6 are also consistent with the notion that direct protonation of the pore at residue 148 elevates some free energy minimum of the K+ ion in the pore. It is very interesting, then, that the effect of protonation of position 148 in the R148H mutant (the positive charge at position 148, 148Δ) on K+ ion conductance or the free energy profile of permeating K+ may be so different in different [K+]o, (Figs. 6 and 7). Because changes of free energy minimum of permeating K+ ion by protonation-deprotonation of the R148H mutant channel pore (manifested as increased open channel noise at pH 7.4) are evident only in high (100 mM) but not in low (10 mM) [K+]o, it seems that 148Δ would have a significant effect on K+ ion permeation only when the pore is “crowded” with permeating K+ ions.

In other words, ion-ion interaction is very likely to be involved in the effect of 148Δ on K+ ion permeation, which would in turn imply simultaneous existence of at least two permeating K+ ions around position 148. We therefore propose that there is a set of ligand groups around position 148 in the external pore mouth to accommodate two permeating K+ ions (Fig. 8). When the ligand at position 148 has an uncharged histidine (Fig. 8B), the ligand may participate in coordinating the partly dehydrated permeating K+, and the set of ligands represents two “complete” binding sites for K+ ions. Because the energy minimum is always low, the Kd and maximal single-channel conductance are both small. However, when the ligand at position 148 is positively charged (Fig. 8C), it can no longer participate in coordinating K+ ions. The sites are now somewhat insufficient to accommodate two K+ ions, and the second K+ would have to adopt a position (site b in Fig. 8A) competing for some coordinating ligands with the first K+ (at site d in Fig. 8A). Both the Kd for loading the second K+ to site b in Fig. 8A and the maximal single-channel conductance would therefore be larger. When [K+]o is low (e.g. ≤ 10 mM) there would be no significant occupancy by the second K+ of site b (whose apparent Kd with site d already occupied by a K+ presumably is ~49 mM, Fig. 6D). The permeation of K+ thus would always happen on an “unenhanced” basis whether position 148 is charged or not (if there is little occupancy of site b by the other K+ ion there would be no significant ion-ion interaction to enhance the exit of the K+ at site d). The unitary conductance and open channel noise level would therefore remain very similar in 10 mM [K+]o, no matter whether it is WT channel or R148H mutant, and no matter whether pHo is 6.0 or 9.0.

The multi-ion nature of inward rectifier K+ channels has been supported by several lines of evidence including anomalous greater fraction effect (15), a flux-ratio exponent of 2.2 (16), steep Vm-dependent block by monovalent ions (17), and Vm and [K+]o-dependent inward rectification (1). However, it is unclear whether and how these multiple K+ ions in the pore interact with each other. In this study, we add a new line of evidence for the multi-ion nature of the Kir2.1 channel pore. Furthermore, we suggest that the interaction between the permeating K+ ions near position 148 is essential for rapid transduction of the K+ ion through the pore and that position 148 likely is directly involved in or close to a set of two K+ binding sites in the external pore mouth. The arginine residue at position 148 is highly conserved in the Kir family, and it is likely that this highly conserved arginine at position 148 will also influence K+ permeation through other inward rectifier K+ channels in a way similar to what is described in this study. The existence of a set of two closely associated K+ binding sites in the external mouth of the Kir2.1 channel is intriguing, because it is very similar to what is described for the t-type Ca2+ channels (18–20). It would be interesting to see whether this is a more general attribute in the molecular design of these cationic channels in the future. In addition to the set of K+ binding sites located near the external mouth of the pore, it has recently been suggested that K+ and Ba2+ may compete for a binding site located in the electrical field in the Kir2.1 channels (11). Further characterization of how K+ ions in these different binding sites interact with each other or with the channel may provide us a more complete picture of the ion permeation process through the pore of the Kir2.1 channel.

Acknowledgments—We thank Drs. Jorge Arreola, Chyuan-Yih Lee, and Kenneth K.-Y. Wu for helpful discussions.

REFERENCES
1. Hille, B. (1992) in Ionic Channels of Excitable Membranes, 2nd Ed., Sinauer, Sunderland, MA
2. Matsuda, H., Saigusa, A., and Irisawa, H. (1987) Nature 325, 156–159
3. Vandenberg, C. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2560–2564
4. White, B. A., Taglialetela, M., Ficker, E., and Brown, A. M. (1994) Nature 371, 246–249
5. Lopatin, A., Makhina, E., and Nicol, C. (1994) Nature 372, 366–371
6. Shieh, R., John, S. A., Lee, J., and Weiss, J. N. (1996) J. Physiol. (Lond.) 494, 363–376
7. Kubo, Y. (1996) Receptors Channels 4, 73–83
8. Kubo, Y., Baldwin, T., Jan, Y., and Jan, L. (1993) Nature 362, 127–133
9. Sabirov, R. Z., Tominaga, T., Miwa, A., Okada, Y., and Okuji, S. (1997) J. Gen. Physiol. 110, 665–677
10. Krapivinovsky, G., Medina, I., Eng, L., Krapivinsky, L., Yang, Y., and Clapham, E. (1998) Neuron 22, 995–1005
11. Shieh, R., Chang, J., and Arreola, J. (1998) Biophys. J. 75, 2313–2322
12. Hamill, O. P., Marty, A., Heher, E., Sakmann, B., and Sigworth, F. J. (1981) Pflugers Arch. Eur. J. Physiol. 391, 85–100
13. Huang, C. L., Feng, S., and Hilgemann, D. W. (1998) Nature 391, 803–806
14. Coulter, K. L., Perier, P., Radeke, C. M., and Vandenberg, C. A. (1995) Neuron 15, 1157–1168
15. Haginawa, S., Miyazaki, S., Krasse, S., and Ciani, S. (1977) J. Gen. Physiol. 70, 269–281
16. Stampe, P., Arreola, J., Perez-Cornejo, P., and Begenisich, T. (1998) J. Gen. Physiol 112, 475–484
17. Haginawa, S., Miyazaki, S., and Rosenthal, N. P. (1976) J. Gen. Physiol. 67, 621–638
18. Kuo, C. C., and Hess, P. (1993) J. Physiol. (Lond.) 466, 629–655
19. Kuo, C. C., and Hess, P. (1993) J. Physiol. (Lond.) 466, 657–682
20. Yang, J., Ellinor, P. T., Sather, W. A., Zhang, J. F., and Tsien, R. W. (1993) Nature 366, 158–161