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The Substates with Mutants That Negatively Charged Aspartate in Position 172 Was Replaced with Positive Charge in Murine Inward Rectifier Potassium Channel (Murine Kir2.1)

I So[2,3], I. Ashmole[1,2], and P.R. Stanfield[1,2]

[1]Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK, [2]Ion Channel Group, Department of Cell Physiology and Pharmacology, University of Leicester, PO Box 138, Leicester LE1 9HN, U.K., [3]Department of Physiology and Biophysics, Seoul National University College of Medicine, 28 Yeonggeon-dong, Jongno-gu, Seoul 110–799, Korea

We have investigated the effect on inducing substate(s) of positively charged residues replaced in position 172 of the second transmembrane domain in murine inward rectifier potassium channels, formed by stable or transient transfection of Kir2.1 gene in MEL or CHO cells. Single channel recordings were obtained from either cell-attached patches or inside-out patches excised into solution containing 10 mM EDTA to rule out the effect of Mg^{2+} on the channel gating. The substate(s) could be recorded with all mutants D172H, D172K and D172R. The unitary current-voltage (I~V) relation was not linear with D172H at pH_i 6.3, whereas the unitary I~V relation was linear at pH_i 8.0. The relative occupancy at S_{LC} was increased from 0.018 at pH_i 8.0 to 0.45 at pH_i 5.5. In H-N dimer, that was increased from 0.016 at pH_i 8.0 to 0.23 at pH_i 5.5. The larger the size of the side chain or pK_a with mutants (D172H, D172K and D172R), the more frequent the transitions between the fully open state and substate within an opening. The conductance of the substate also depended upon the pKa or the size of the side chain. The relative occupancy at substate S_{LC} with monomer D172K (0.50) was less than that in K-H dimer (0.83). However, the relative occupancy at substate with D172R (0.79) was similar to that with R-N dimer (0.82). In the contrary to ROMK1, positive charge as well as negative charge in position 172 can induce the substate rather than block the pore in murine Kir2.1. The single channel properties of the mutant, that is, unitary I~V relation, the voltage dependence of the mean open time and relative occupancy of the substates and the increased latency to the first opening, explain the intrinsic gating observed in whole cell recordings.

Key Words: Potassium channel, Substate, Channel gating

INTRODUCTION

An amino acid located in the second transmembrane has been known to be involved with an intrinsic gating in inward rectifier potassium channel. Some investigators tried to change the amino acid to positively charged amino acids and see the effects of such mutations on the intrinsic gating (Lu & MacKinnon, 1994; Shyng, Ferrigni & Nichols, 1997). Such mutations induced a different current-voltage relationship from that of wild type in ROMK1 (Lu & MacKinnon, 1994), whereas slight Rb efflux was recorded in K (ATP) channel (Shyng, Ferrigni & Nichols, 1997). In the previous paper (So et al, 2003), we showed that the positive charge itself can induce an intrinsic gating independent of polyamine in murine inward rectifier potassium channel, Kir2.1. The intrinsic gating can be explained by the late latency to the first opening of the channel and frequent transition between the opening and substate, or between substates. In a mutant of ROMK1 (N171H), histidine residue renders the channel sensitive to pH (Lu & MacKinnon, 1994). They suggested that a proton binds to a histidine and induces a nonconducting state (Lu & MacKinnon, 1994). However, when mutants in murine Kir2.1, where the negatively charged aspartate was replaced with positively charged amino acids such as arginine, lysine and histidine, were expressed, they can pass through current in murine Kir2.1. In this paper, we show that the positive charge can induce substates in Kir2.1 rather than block the channel completely in murine Kir2.1, which might be the mechanism of the intrinsic gating in mutant Kir2.1, D172H, D172K and D172R. A preliminary account of this work has been given (So et al, 2000).

METHODS

Molecular biological methods

The coding region of Kir2.1 cDNA (Stanfield et al, 1994)

Corresponding to: Peter R. Stanfield, Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK. (Tel) +44-24-765-72503, (Fax) +44-24-765-23701, (E-mail) pstanfield@bio.warwick.ac.uk

ABBREVIATIONS: IRK, inward rectifier postassium channel; ROMK, inward rectifier K channel form the renal outer medulla.

was cloned into the expression vector pEV3 as an EcoRI/BglII fragment for MEL (murine erythroleukaemia) cells or pcDNA3 as an EcoRI/XhoI fragment for CHO (Chinese hamster ovary) cells. Substitution mutations were generated by oligonucleotide-directed in vitro mutagenesis. Mutations were verified by DNA sequencing of the entire Kir2.1 cDNAs. Ion channels were expressed in MEL stably with electroporation, or CHO cells transiently using the pTx^{TM} cationic lipid transfection reagent (Invitrogen) according to manufacturers' instructions. The selection of G418-resistant colonies and induction of expression in MEL cells were as described previously (Stanfield et al, 1994). As a transient transfection marker, plasmid DNA containing the cDNA for Green Fluorescent Protein (Molecular Probe) was cotransfected with the Kir2.1 cDNAs. The channel properties of ion channels expressed in MEL or CHO cells, including activation time constant, current-voltage relation, conductance-voltage relation and single channel currents, were similar.

For some experiments, we made covalently linked tandem dimer (N-H, N-K or N-R) and tetramer(N-N-N-H) as described previously (Dart el al., 1998).

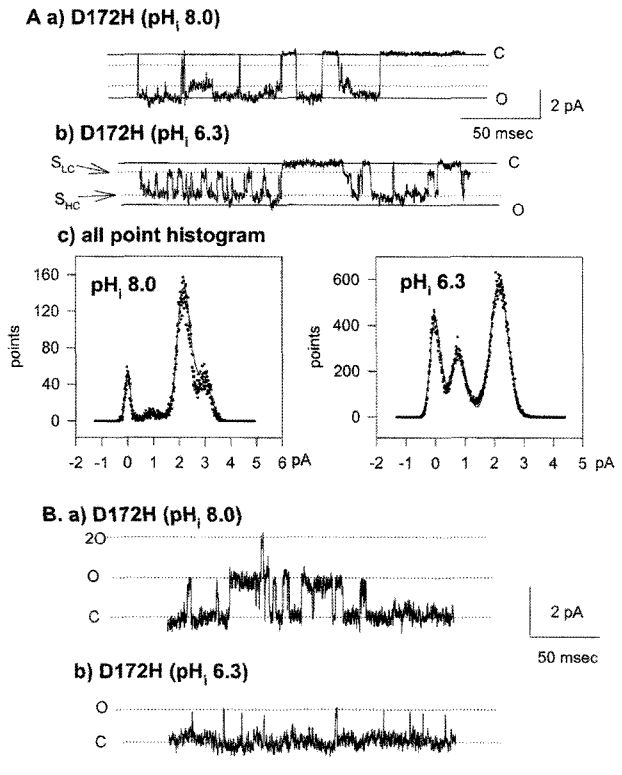

Fig. 1. The single channel recordings with D172H. A. Single channels were recorded from an inside-out patch in 140 mM KCl solution with different internal (bath) pH values. O means the open state and C the closed state. There were two substates, called as S_{HC} and S_{LC}. Single channel currents were recorded at −140 mV. External (pipette) solution was pH 7.2. In (c), only 2048 points are used for all-point histogram to show the open state as well as both substates. B shows the effect of pH_i on the outward current with D172H. Intracellular proton causes the open time short and the amplitude to decrease.

Electrophysiological methods

Membrane currents were measured by single channel recording, using an Axopatch 200A amplifier. Records were filtered at 2 kHz (−3 dB, 8-pole Bessel), digitized at 10 kHz using Digidata 1200 or TTL interface (Axon Instruments, Foster City, CA, USA), and analysed on a 486 computer. Intracellular solution contained (mM): Hepes, 10; EDTA, 10; pH adjusted to 7.2 with KOH; KCl added to bring $[K^+]$ to 140 mM. Extracellular solution contained (mM): KCl, 140 or 200; $CaCl_2$, 2; $MgCl_2$, 2; Hepes buffer (pH 7.2), 10. For single channel recording at cell-attached or inside-out patch mode, we used the intracellular solution of 140 mM K^+ for the bath solution and 140 mM or 200 mM K^+ for the patch solutions. Experiments were carried out at room temperature (20~23°C).

RESULTS

The substate with mutant D172H

We recorded single channel currents at inside-out mode with D172H under the condition of 140 mM $[K^+]_o$ and $[K^+]_i$. With D172H at pH_i 8.0, we could record two substates with a higher conductance (S_{HC}) and a lower conductance (S_{LC}) at −140 mV (Fig. 1). In one cell expressing D172H mutant, we could record fully open state (O) for a relatively long time. The current amplitudes for each states from all-point histogram were 3.0±0.2 pA, 2.2±0.2 pA and 0.96±0.3 pA for O, S_{HC} and S_{LC} states, respectively. However, when we obtained an all-point histogram for more than 20 seconds, the O state could not be distinguished from the S_{HC} state. In most cells, it was difficult to distinguish the O state from the S_{HC} state. Even at pH_i 8.0, the mutation of aspartate to histidine can induce two substates.

As intracellularly applied proton was increased to make histidine more positively charged, there were three changes in channel properties. First, proton induced more frequent transitions between the substates within an opening (Figs. 2A and 3C). We recorded single channel currents under the condition of 200 mM $[K^+]_o$ and 140 mM $[K^+]_i$. The number of transitions between S_{HC} and S_{LC} within an opening increased from 0.93±0.12 (n=4) at pH_i 8.0, 3.8±0.3 (n=4) at pH_i 7.2 to 16.1±0.5 (n=4) at pH_i 6.3 at −100 mV. Secondly, there was the change in the shape of unitary current- voltage (I~V) relationship (see So et al, 2003, Fig. 5B). The I~V relationship was linear at pH_i 8.0, whereas that at pH_i 6.3 was not. Especially the change on outward current makes the I~V relationship look like non-linear. The open time of outward current was brief, compared with that at pH_i 8.0 (Fig. 1B). The conductances of the substates, S_{HC} and S_{LC}, were 24.5±1.4 pS (n=3) and 7.3±0.3 pS (n=5) at pH_i 8.0, respectively. The slope conductance at pH_i 6.3 at the range from −60 mV to −120 mV was 34.1±2.8 pS (n=4). Thirdly, there were the changes in the relative occupancy and an arithmetic mean dwelling time. We manually measured the dwelling time at both S_{HC} and S_{LC} to get a relative occupancy and an arithmetic mean dwelling time (Figs. 2 and 3). The arithmetic mean dwelling time at S_{HC} decreased, as pH_i decreased from 93.5±3.3 msec at pH_i 8.0 (n=4) to 2.7±0.2 msec at pH_i 5.5 (n=3) (Figs. 2 and 3A). The arithmetic mean dwelling time at S_{LC} was similar at the different pH_is (Fig. 3A). The relative occupancies at S_{HC} decreased, as pH_i decreased from 0.983±0.005 (n=4) at pH_i

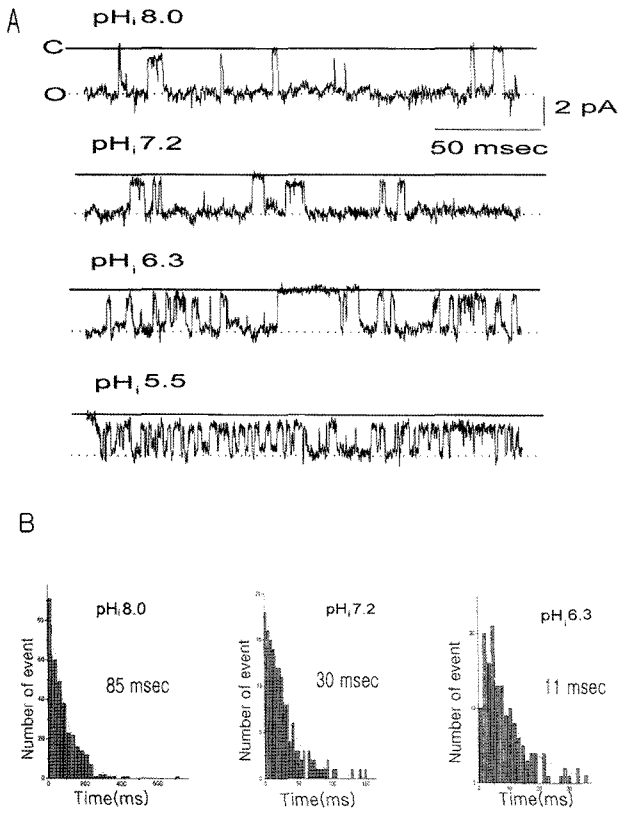

Fig. 2. The effect of intracellular proton on the channel properties. A. Single channels were recorded from an inside-out patch in 200 mM KCl solution with different internal (bath) pH values. O means the open state and C the closed state, respectively. Single channel currents were recorded at −100 mV. External (pipette) solution was pH 7.2. B shows dwelling time histograms. Transitions between the two substates were determined with a manual 50% threshold criterion and cut-off times of 0.28 msec for 2 kHz data (1.65 times the filter rise time). Time bins of histograms are 25 ms for pH$_i$ 8.0, 4 ms for pH$_i$ 7.2 and 1.4 ms for pH$_i$ 6.3. The histograms at different pH$_i$ were fitted with a single exponential function with the time constants indicated.

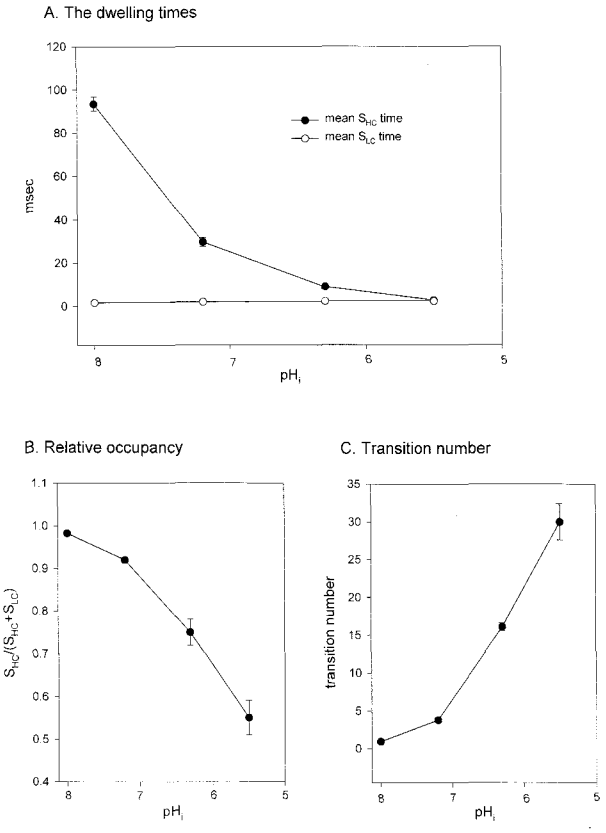

Fig. 3. The effect of intracellular proton on the channel properties. A shows dwelling times at different pH$_i$. The arithmetic mean dwelling time (n=3~4) is plotted against pH$_i$. In B, the relative occupancy was obtained by $S_{HC}/(S_{HC}+S_{LC})$. S_{HC} and S_{LC} indicate the total dwelling time for each substate. The arithmetic mean of the relative occupancy (n=3~4) is plotted against pH$_i$. In C, the transitions between the two substates were counted manually. The arithmetic mean of the transition numbers (n=3~4) is plotted against pH$_i$.

8.0 to 0.55±0.04 (n=3) at pH$_i$ 5.5 (Fig. 3B). On the other hand, the relative occupancies at S_{LC} changed to opposite direction from 0.0175±0.005 at pH$_i$ 8.0 to 0.45±0.04 at pH$_i$ 5.5. These results might come from either the protonation-deprotonation in histidine residues or the presence of the positive charges, which they carry. The proton did not block the channel pore unlike in ROMK, but induced substates. Since protons are small and there is a wide space with the pore in Kir2.1 (see Lu et al, 1999), it does not seem that protons have much effect on conductance by physically blocking the channel (but see ROMK1). Thus, it would seem likely that the positive charge is the most important factor in the induction of substates by proton. The fact that conductance of the substate S_{HC} increases as proton concentration increases, together with non-linear I/V curves at pH$_i$ 6.3, suggests that protonated histidine itself has its own effect on the permeation of potassium ions.

Next, we tested how many residues are required to induce the substate. We could express mutants of D172H containing 2 (H-N-H-N; dimer) and 1 histidine residue(s) (N-N-N-H; tetramer). Even with tetramer containing one histidine, the substate can be recorded at cell-attached mode under the condition of 140 mM $[K^+]_o$ and $[K^+]_i$ (Fig. 4A). In the beginning, we assumed that one proton binds in the higher conductance substate and two bind in the lower conductance substate. However, we could record only the lower conductance state instead of the higher conductance state. Dimer and tetramer seem to have only one substate (S_{LC}) (see also D172K and N-K dimer in Fig. 6). The conductance at the open state with dimer or tetramer was similar to that of S_{HC} with monomer. The conductances at substate S_{LC} were 6.5±0.2, 7.3±0.5 and 6.2±0.6 (n=3) for tetramer, dimer and monomer, respectively (Fig. 4B). They were not significantly different ($P>0.05$), although it seems that the conductance with dimer is greater than that with tetramer or monomer. Decreasing the number of histidine residue in mutants causes 1) decrease of the number of the substate and 2) decrease of the conductance of the open state.

The arithmetic mean dwelling time at fully open state or S_{HC} also increased from 27±2 msec (n=3) with monomer, 44±4 msec (n=3) with dimer, to 56±4 msec (n=3) with tetramer at −160 mV (Fig. 8A). The arithmetic mean dwelling time at substate changed to opposite direction

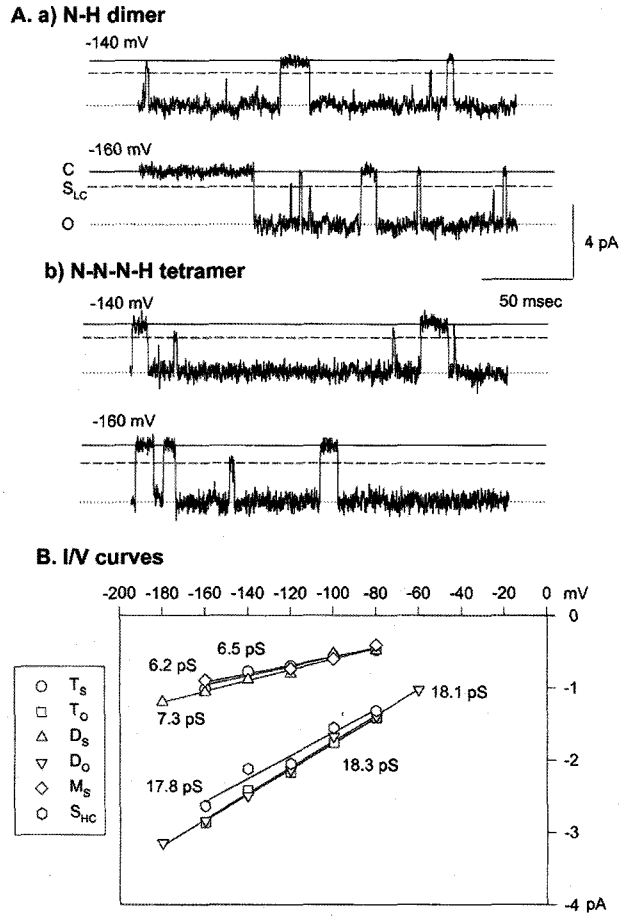

Fig. 4. The single channel currents at different voltages in H-N dimer and H-N-N-N tetramer. A shows the raw current traces at voltages, −140 mV and −160 mV. O means the open state, S_{LC} a substate and C the closed state. Single channels were recorded from a cell-attached mode in 140 mM KCl solution. B shows I/V curves in dimer and tetramer. Each symbol is the mean (n=3) of measurements made in separate patches.

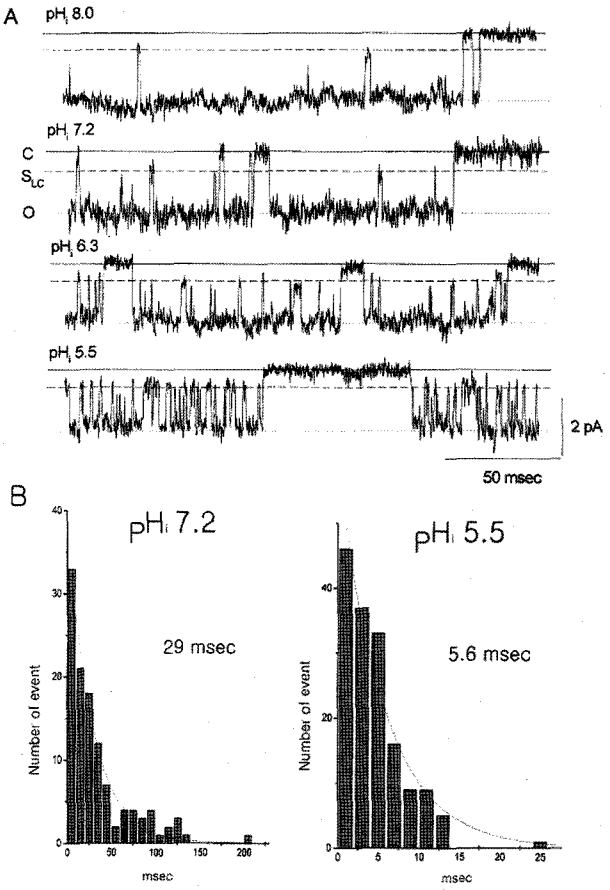

Fig. 5. The effect of intracellular proton on the channel properties in H-N dimer. A. Single channels were recorded from an inside-out patch in 140 mM KCl solution with different internal (bath) pH values. S_{LC} means the substate with lower conductance, O the open state and C the closed state. Single channel currents were recorded at −100 mV. External (pipette) solution was pH 7.2. B shows dwelling time histograms. Transitions between the two substates were determined with a manual 50% threshold criterion and cut-off times of 0.28 msec for 2 kHz data (1.65 times the filter rise time). Time bins of histograms are 10 ms for pH_i 7.2 and 2 ms for pH_i 5.5. The histograms at different pH_i were fitted with a single exponential function with the time constants indicated.

from 1.31±0.10 (n=3) with monomer, 1.25±0.06 (n=3) with dimer, to 0.95±0.04 (n=3) with tetramer. The mean dwelling time at fully open state or S_{HC} approached the value at pH_i 8.0 from the value at pH_i 7.2 as the histidine number decreased from 4 to 1. The relative occupancy at fully open state or S_{HC} increased from 0.959±0.002 (n=3) with monomer, 0.982±0.002 (n=3) with dimer to 0.986±0.003 (n=3) with tetramer (Fig. 8B). The relative occupancy at substate changed to opposite direction from 0.041±0.002 (n=3) with monomer, 0.0180±0.002 (n=3) with dimer to 0.0137±0.003 (n=3) with tetramer. The number of transitions within an opening decreased from 5.2±0.4 (n=4) with mononer, 2.4±0.3 (n=4) with dimer to 2.0±0.4 (n=4) with tetramer at −160 mV. These values were quite similar to the value at pH_i 7.2, regardless of the number of histidine residue.

The number of histidine residues determined the pH_i sensitivity of channel properties in mutant D172H. The pH_i sensitivity in H-N dimer was less than in D172H monomer. With H-N dimer, we recorded single channel currents in 140 mM $[K^+]_o$ at −120 mV. The arithmetic mean dwelling time at fully open state decreased, as pH_i decreased from 36.5±3.3 msec at pH_i 7.2 (n=4) to 5.7±0.2 msec at pH_i 5.5 (n=3) (Fig. 5). The relative occupancies at S_{LC} increased, as pH_i decreased; 0.016±0.003 (n=3) at pH_i 8.0, 0.019±0.002 (n=3) at pH_i 7.2, 0.12±0.02 (n=3) at pH_i 6.3, and 0.23±0.03 (n=3) at pH_i 5.5. These results suggest that more intracellular protons are needed in dimer than monomer to show the similar effect.

The substate with mutants D172K and D172R

If we assume that the substate occurs as histidines are protonated, and that the transitions occur because of the rapid bindings and unbindings of protons with histidine residues within the pore, we can expect the following. The relative occupancy at the substate should approach to the relative occupancy of around one with D172K (pK_a of 10.80) or D172R (pK_a of 12.48). First, we recorded single channel currents at cell-attached mode with D172K (Fig. 6). With

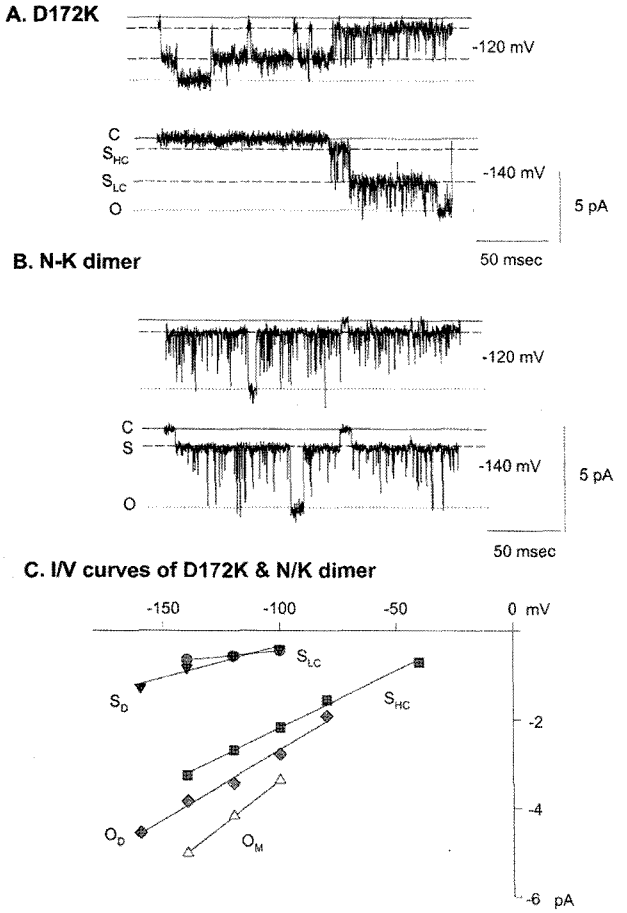

Fig. 6. The single channel currents in D172K (A) and K-N dimer (B). Single channels were recorded from a cell-attached patch in 200 mM KCl solution. S_{HC} and S_{LC} mean the substate with higher conductance and lower conductance, respectively. O means the open state, S the substate and C the closed state. Single channel currents were recorded at −120 mV and −140 mV. External (pipette) solution was pH 7.2. C shows I/V curves in dimer and tetramer. Each symbol is the mean (n=3~4) of measurements made in separate patches.

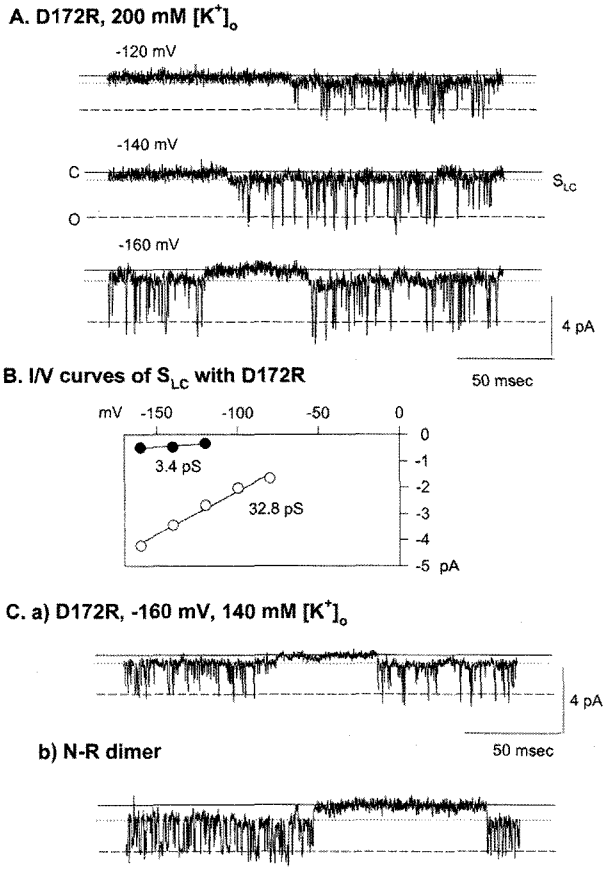

Fig. 7. The single channel currents in D172R (A) and R-N dimer (C). In A, single channels were recorded from a cell-attached patch in 200 mM KCl solution. S_{LC} means the substate with a lower conductance. O means the open state and C the closed state. Single channel currents were recorded at −120 mV, −140 mV and −160 mV. External (pipette) solution was pH 7.2. B shows I/V curves in dimer and tetramer. Each symbol is the mean (n=3~4) of measurements made in separate patches. In C, raw current traces were compared between D172R and R-N dimer at the same membrane potential (−160 mV) in 140 mM KCl solution.

D172K, 2 substates could be recorded and the fully open state was brief like with D172H (Fig. 6A). We obtained the amplitude of the open state by measuring an average value during a relatively short time when the channel stayed at the open state. The fully open state and substates with a higher conductance (S_{HC}) and a lower conductance (S_{LC}) have conductances, 38.6±1.4, 25.4±1.2 pS and 5.3±0.6 pS (n=4) at the range from −80 mV to −160 mV, respectively (Fig. 6C). We could not record outward currents at inside-out modes. The numbers of transitions were 24.8±0.6 (n=3) at −140 V. Even D172K that is thought to have positive charge at the physiological condition, like at cell attached mode or pH_i 7.2, the relative occupancy at substate (S_{LC}) was 0.50±0.07 (n=3), not almost 1.0 as expected from the previous investigation. These results suggest either that the pK_a of the side chain is perturbed -being lower than those of the free amino acids- or that positive charge in lysine induces such substates.

With D172R, at least one substate could be recorded and the conductance of the open state and substate were 32.8± 0.9 pS (n=3) at the range from −80 mV to −160 mV and 3.4±0.5 pS (n=3) at the range from −120 mV to −160 mV, respectively (Fig. 7). The numbers of transitions were 29.8 ±2.3 (n=3) at −140 mV. The order at the number of transitions between the fully open state or S_{HC} and the substate (S_{LC}) was D172R≅D172H at pH_i 5.5 (29.5±2.4, n=3)> D172K>D172H at pH_i 6.3.

We could also make dimers of D172K (N-K) and D172R (N-R) with D172N (Figs. 6 and 7). Even in dimers (N-K and N-R dimers) where there are two positive charges compared with four in monomer, the substate was observed even though the number of substate decreased by 1 in case of D172K but the same in D172R. With K-N dimer, the fully open state and substates with a lower conductance (S_{LC}) have conductances, 30.7±0.4 at the range from −40 mV to −160 mV and 5.4±0.5 pS (n=3) at the range from −100m V to −160 mV, respectively. The substate with a lower conductance (S_{LC}) with N-R dimer, however, has a higher conductance (0.90±0.01 pA, n=4) than that of monomer (0.66±0.02 pA, n=3) at −160 mV (Fig. 7C). The

relative occupancy in substate (0.83±0.02, n=3) with N-K dimer was greater than that with monomer, which did not suggest the protonation-deprotonation hypothesis. In addition, the relative occupancy in substate (0.82±0.03, n=3) with N-R dimer was similar to that with monomer (0.80 and 0.78). However, the relative occupancy at S_{LC} state (0.22 at pH_i 5.5) with H-N dimer was decreased. These results suggest that the positive charge itself as well as simple protonation-deprotonation is important for inducing the substate and the transitions.

The voltage dependence of channel properties in mutants

Finally, the voltage dependence of mean open time and the relative occupancy of the open state or the substate S_{HC} are shown in Fig. 8. The mean open time of the open state or the substate S_{HC} was increased in mutants of D172H, as the membrane potential became hyperpolarized (Fig. 8A). On the other hand, the mean open time in mutants D172K and D172R was shorter and voltage-independent. Thus, the mean open time depended upon the pKa values of amino acid residues. The higher pKa value induced briefer mean open times. Interestingly, the mean open time also increased as the number of histidine decreased in mutants of D172H at all membrane potentials. The relative occupancy of the open state or the substate S_{HC} was voltage dependent in mutants D172H at pH_i 6.3, D172K and D172R (Fig. 8B). The voltage dependency was weak in mutants of D172H, such as, monomer, dimer and tetramer, in cell-attached mode.

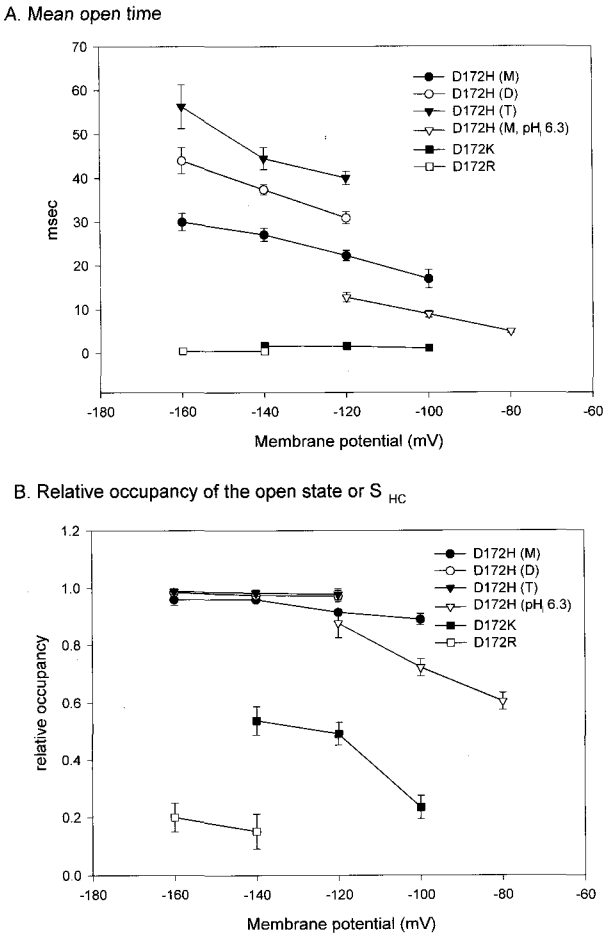

Fig. 8. The voltage-dependence of mean open time and relative occupancy in mutants. A shows the mean open time among mutants (n= 3~4), monomer, H-N dimer, H-N-N-N tetramer, D172K and D172R. The mean open time tends to increase as the membrane potential became hyperpolarized. B shows the relative occupancy among mutants (n= 3~4), D172H monomer, H-N dimer, H-N-N-N tetramer, D172K and D172R. The relative occupancy of the open or S_{HC} state tends to increase as the membrane potential became hyperpolarized.

DISCUSSION

The transitions between two substates or substate and open state might result from either the protonation-deprotonation in histidine residues or the presence of the positive charges, which they carry. At least three results support the protonation-deprotonation hypothesis. First, transition number, mean open time and relative occupancy of the substate S_{HC} or open state decreased as pHi decreased. These results are similar to that obtained in cyclic nucleotide gated (CNG) channel (Root & MacKinnon, 1994) or voltage-dependent Ca^{2+} channel (Chen et al, 1996), as external pH was changed. In the case of latter channels, proton binds with glutamate or aspartate. With D172H, proton binds with histidine, which in turn causes histidine to get a positive charge. There was another difference at pK_a value among them (pK_a of 7.4 with D172H from So et al (Fig. 6); 7.58 with CNG channel (Root & MacKinnon, 1994); around 8.5 with L-type Ca^{2+} channel (Chen et al, 1996)). The difference might come from either the site of D172 within the pore deep from the cytoplasmic side of the membrane or the electrostatic repulsion from the positive charge in histidine residues. Secondly, mean open time and relative occupancy at the substate S_{HC} or open state were voltage-dependent. Hyperpolarization increased the mean open time and the relative occupancy. The fraction of unblocked current measured at different proton concentrations, relative to that measured at pH_i 9, was plotted as a function of pH_i and the pK_a values ($-\log K_i$) are plotted as a function of membrane potential (So et al, 2003, Fig. 6). The voltage dependence suggests that a proton crosses 52% of the transmembrane voltage, when it travels from the intracellular solution to its binding site in the pore (cf. In ROMK, δ was 0.4 or 0.44 depending on $[K^+]_o$). Thirdly, the number of histidine in the mutant determined the number of the substates.

Three results support other hypothesis that positive charge in histidine, lysine and arginine results in the transition between the substates, or the substate and the open state. First, the proton did not block the channel pore unlike in ROMK, but induced substates. Since protons are small and there is a wide space with the pore in Kir2.1 (see Lu et al, 1999), it does not seem that protons have much effect on conductance by virtue of physically blocking the channel (but see ROMK1). The protons also changed the shape of I/V curves to non-linear form at pH_i 6.3. Depending upon the size of side chain or pK_a value of the residues, the amino acid residues determined the unitary conductance of the common substate among the mutants and the transitions between the two states. Secondly, conductance of the substate S_{HC} increases, as proton

concentration increases from pH_i 8.0 to pH_i 6.3. Thirdly, there is the substate S_{LC}, not the substate S_{HC}, in tetramer mutant. This result does not support the hypothesis, as suggested in CNG channel (Root & MacKinnon, 1994) or voltage-dependent Ca^{2+} channel (Chen et al, 1996), that the first proton binds to the histidine and induce S_{HC} and the second proton then binds to another histidine and induce the substate S_{LC}. Lu et al. (1999) showed sulfhydryl-specific modifying reagent, containing positive charge (MTSET), could induce only substate, but there was no transitions because of its fixed charge in MTSET. Thus, it would seem likely that the positive charge is another important factor in the induction of substates by proton, and protonated histidine itself has its own effect on the permeation of potassium ions.

Inward rectifier potassium channels (IRKs) have been shown to adopt substates of conductance in ventricular cells from guinea pig heart. The substates could be induced by external monovalent cations in the inward direction, like Cs^+ and Rb^+, and internal divalent cations in the outward direction, like Mg^{2+} and Ca^{2+}. Recently, Oishi et al. (1998) showed that Mg^{2+}-induced subconductance states come from the negative charge in position 172 like D172 and D172E, whereas there was no substate in D172Q and D172N. They also showed that channels from tandem tetramers of IRK1 with one and two D172N mutant subunits mainly showed sublevels with two-thirds amplitude, whereas those from tetramers with three D172N mutant subunits showed no sublevels. However, Korchev et al. (1997) have shown that such substates could be induced by changes in ionization of fixed charges within a channel or pore. In our experiments, we showed that even positive charge can induce the substates, and that only one positive charge can induce a substate in Kir2.1. Our results agree well with those from the purely synthetic, nonbiological membrane and *S. aureus* toxin (Korchev et al, 1997). They suggested that a fluctuation of surface charge could induce open state in the low conductance state. However, considering that the conductance in *S. aureus* toxin was maximal at low pH, the mechanism of substates in our case seems different from those in toxin.

In conclusion, the intrinsic gating in mutants D172H, D172K and D172R could be explained by the following. The positive charge as well as the protonation-deprotonation induces the substate. The relative occupancy of substate depends upon the membrane potentials in favouring the large current in the hyperpolarized membrane potentials. The unitary I/V curve also favours the large current in the hyperpolarized membrane potentials. The activation phase comes from the late latency to the first opening during hyperpolarizing pulses and the frequent transitions between two substates or the substate and the open state.

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