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Intrinsic Gating in Inward Rectifier Potassium Channels (Kir2.1) with Low Polyamine Affinity Generated by Site Directed Mutagenesis

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We have studied mutant forms of Kir2.1 in which an aspartate residue (D172), important for gating by intracellular polyamines, is replaced by one of three basic residues (Arg, Lys or His). Such channels are highly selective for K^+, but show inward rectification that is a shallow function of voltage compared with that found in wild type. This inward rectification occurs with a reduced affinity for spermine and persists in the absence of polyamines. Though the unitary current-voltage relation shows some inward rectification, it is insufficient to account for that seen under whole cell recording. Channels open and shut under single channel recording, and changes of P_{open} appear to generate inward rectification. In D172H, the reduction in affinity for spermine is greater when His is protonated at low pH_i. The effective valency for spermine is reduced from 3.09 ± 0.07 in wild type to 1.95 ± 0.09 in D172H at pH_i 6.3. In the presence of dual mutants of Kir2.1, where E224 is also replaced, spermine affinity becomes undetectable. However, channels still show inward rectification and open and shut under hyper- and depolarisation, respectively. We suggest that Kir2.1 channel are able to undergo conformation changes; these changes may be important physiologically in generating inward rectification, the normal parameters of which are set by the binding of polyamines such as spermine.

Key Words: Potassium channel, Channel gating, Site-directed mutagenesis

INTRODUCTION

Strong inward rectifier K^+ channels such as Kir2.1 (IRK1) play a key role in setting the resting membrane potential and in regulating excitability in heart, skeletal muscle, and many other cell types (see, for example, Doupnik et al, 1995; Stanfield et al, 2002). Strong inward rectifier potassium channels are believed to be gated by the plugging of the pore by intracellular Mg^{2+} (Matsuda et al, 1997; Vandenberg, 1997) and polyamines such as spermine (Ficker et al, 1994; Lopatin et al, 1994; Fakler et al, 1995). Such gating through channel blockage by intracellular cations leads to channel open state, probability being higher at more negative voltages and, since permeant K^+ competes with blocking cations, with higher $[K^+]_o$.

In Kir2.1, channel gating is regulated by an aspartate residue (D172) in the second (M2) of two transmembrane domains (Stanfield et al, 1994; Taglialatela et al, 1994) and glutamate residues in the C-terminus (E224: Taglialatela et al, 1995; Yang et al, 1995; E299: Kubo & Murata, 2001). These residues appear to regulate channel gating by acting as the receptor or binding site(s) for intracellular polyamines, since their mutation reduces polyamine affinity (Taglialatela et al, 1995; Yang et al, 1995; Kubo & Murata, 2001).

However, Kir2.1 channels still exhibit weak rectification after insertion in lipid bilayers in the absence of Mg^{2+} or polyamines (Aleksandrov et al, 1996), and Shieh et al. (1999) have reported an intrinsic, pH-sensitive gating mechanism in Kir2.1 expressed in oocytes. Thus, channels may possess an intrinsic gating process whose physiological properties are set by an interaction with intracellular polyamines. In support of such a hypothesis, Lee et al. (1999) have shown that certain polyamine spider toxins (argiotoxin, philanthotoxin), which possess a hydrophobic head group, can inhibit the high affinity binding of spermine without necessarily causing channel blockage. Further, spermine can bind to the C-terminus, screen negative charges associated with acidic residues there and so reduce unitary current without necessarily causing channel blockage (Xie et al, 2002). Lee et al. (1999) suggest that polyamines bind, possibly to the C-terminus, to form a gating complex that subsequently shuts the pore.

We have tested a similar hypothesis by reducing

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ABBREVIATIONS: MEL, murine erythroleukaemia; CHO, chinese hamster ovary; GFP, green fluorescent protein.

polyamine affinity by making mutations of D172 that involve its replacement with a basic amino acid residue, histidine (H), lysine (K), or arginine (R). Such channels show gating that is only weakly dependent on intracellular polyamines. They show inward rectification in the absence of polyamines. A further reduction in polyamine affinity was achieved in double mutants made by also replacing Glu 224 by Gly or Gln. This procedure resulted in channels whose gating and inward rectification were completely independent of intracellular polyamine. The results support the hypothesis that Kir2.1 channels can show intrinsic gating that can generate inward rectification, but this intrinsic rectification is only weakly dependent on voltage.

Preliminary accounts of this work have been given (So et al, 1999; 2000).

METHODS

Molecular biological methods

Both mammalian cell lines and *Xenopus* oocytes were used for expression. For expression in murine erythroleukaemia (MEL) cells, the coding region of Kir2.1 cDNA (Stanfield et al, 1994) was cloned into the expression vector pEV3 using an *EcoRI*/*BglII* fragment. Ion channels were stably expressed in MEL cells using electroporation. The selection of G418 resistant colonies and the induction of expression in MEL cells were as described before (Shelton et al, 1993).

For expression in chinese hamster ovary (CHO) cells, an *EcoRI*/*XhoI* fragment was cloned into pcDNA3. Channels were transiently expressed in CHO cells using the pTx™ cationic lipid transfection reagent according to the instructions of the manufacturer (Invitrogen). As a marker for transient transfection, plasmid DNA containing the cDNA for enhanced green fluorescent protein (EGFP; Molecular Probes) was cotransfected with the Kir2.1 cDNAs.

For oocyte expression, Kir2.1 cDNA was cloned into the expression vector pBF (a generous gift from Dr B Fakler) as an *EcoRI*/*XhoI* fragment. Complementary RNAs for the Kir2.1 channels were synthesised *in vitro* from a *MluI*-linearised plasmid using SP6 polymerase (Ambion, USA). Oocytes were obtained from *Xenopus laevis* (XenopusOne, Dexter MI) using methods, described previously, that accord to the highest standards of institutional guidelines (Park & MacKinnon, 1995; Ha et al, 2000). Oocytes were injected with approximately 50ng of RNA, and the injected oocytes were incubated at 18°C for 1~7 days in ND96 solution containing (mM): HEPES, 5; NaCl, 96; KCl, 2; $CaCl_2$, 1.8; $MgCl_2$, 1. NaOH was used to adjust the pH to 7.6 and gentamycin was added to the solution to a concentration of 50 μmg.ml^{-1}.

Substitution mutations were generated by oligonucleotide-directed *in vitro* mutagenesis either by the method of Taylor et al. (1985) or by the method of Kunkel (1985). Mutations were verified by full length sequencing of the Kir2.1 cDNAs.

Electrophysiological recording

An Axopatch 200A amplifier (Axon Instruments, Union City, CA, USA) was used to measure membrane currents by either whole cell or single channel recording modes of the patch clamp. Recording of channel currents from oocytes used macropatches. Records were filtered at 2 or 5kHz (-3dB, 4-pole Bessel), digitised using a Digidata 1200 or TTL interface (Axon Instruments), and analysed on a 486 computer using a software suite developed by NW Davies (Department of Cell Physiology and Pharmacology, University of Leicester).

For whole cell recording from mammalian cell lines, patch pipettes were filled with a Mg^{2+}-free internal solution containing HEPES, 10mM; EDTA, 10mM; pH adjusted to 7.2 with KOH; KCl, added to bring [K^+] to 140mM. HEPES was also used as the buffer in some experiments at pH 6.3 and 8.0. Otherwise, we used PIPES (pH 6.3 and 7.2); TAPS (pH 9.0); or MES (pH 5.5, 6.3 & 7.2)—all at 10 mM with pH adjusted with KOH and with final [K^+] brought to 140 mM with KCl. The extracellular solution contained (mM): KCl, 70; NaCl, 70; $CaCl_2$, 2; $MgCl_2$, 2; HEPES buffer (pH 7.2), 10. In some experiments, $[K^+]_o$ was reduced by replacement of KCl by NaCl or raised by replacement of NaCl by KCl.

For single channel recording from mammalian cells, using the inside out mode, the bath contained internal solution (HEPES, 10 mM; EDTA, 10 mM; pH adjusted to 7.2 with KOH; final [K^+] to 140 mM with KCl). The patch pipette solution contained 140 or 200 mM K^+ (KCl 140 or 200 mM; $CaCl_2$, 2 mM; $MgCl_2$, 2 mM; HEPES buffer 10 mM; pH7.2).

Unitary current amplitudes were measured either directly from the traces or by forming amplitude histograms of selected sections of recording containing clear single open and closed current levels. Gaussian distributions were fitted to the amplitude histograms and the means of these distributions were used to calculate unitary current. Open state probability, open and closed times, and the latency to the first opening after a voltage step were analyzed with a suite of programs developed by NW Davies using the AxoBASIC library. The open state probability (P_{open}) was calculated either from amplitude histograms or by using cursors set at 50% of open according to the equation

$$P_0 = \sum_{j=1}^{N} \frac{jt_j}{NT}$$

In this equation, t_j is the time when j channels are open simultaneously during a recording that lasts for time T; N is the total number of channels in the patch.

For macropatch recording from *Xenopus* oocytes, the bath solution contained 140 mM K^+ (HEPES 10 mM; EDTA, 10 mM; pH adjusted to 5.5, 6.3, 7.2, 8.0, or 9.0 with KOH; KCl to bring [K^+] to 140 mM). In a few experiments, to check whether certain of the phenomena described could be due to blockage by HEPES (see Guo & Lu, 2000), we used phosphate buffer, replacing HEPES with 10 mM K_2HPO_4 and KH_2PO_4 in appropriate ratio. The pipette solution contained KCl, 140 mM; $CaCl_2$, 2 mM; $MgCl_2$, 2 mM; HEPES buffer (pH 7.2). For macropatch recordings, the membrane was held at 0 mV and ramped from −100 mV to 100 mV over 1 s.

Experiments were carried out at room temperature, 20~23°C.

RESULTS

Effects of mutations of Asp172

Previous reports have suggested that substitution by a basic residue of Asp172 in Kir2.1 or its equivalent in Kir6.2 (N160) results in channels that are non- or only poorly

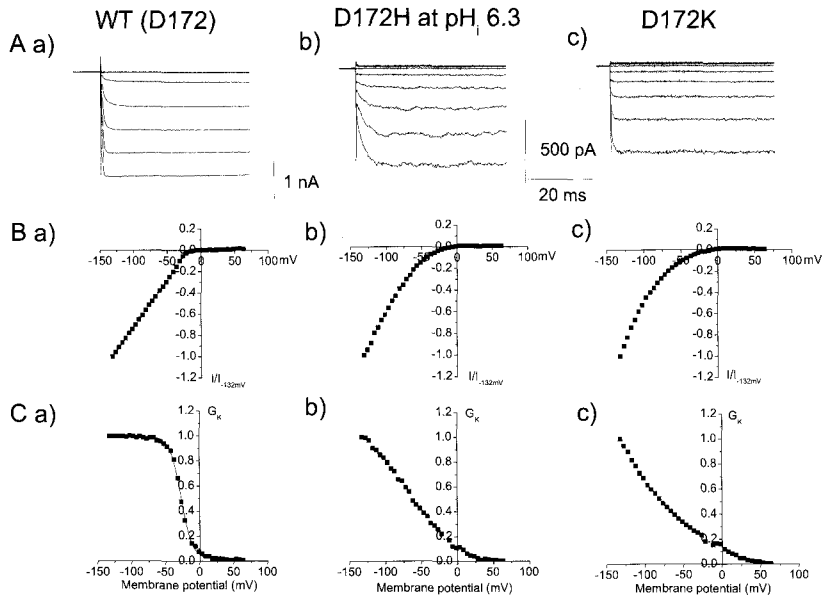

Fig. 1. Inward rectification in wild-type and mutant forms of Kir2.1. A, membrane currents recorded from MEL cells transfected with wild type (WT, a) or mutant (D172H at pH_i 6.3, b; D172K, c) forms of Kir2.1. $[K^+]_o$=70 mM; $[K^+]_i$=140 mM. The Lys residues in D172K are likely to be positive at pH_i 7.2. Whole cell currents were recorded with patch clamp techniques. Voltage pulses were applied in 5mV increments from 63 mV to 132 mV from a holding potential of −17 mV, equivalent to the potassium equilibrium potential (E_K). Currents are illustrated in 20 mV steps up to 80 mV positive and down to 100 mV negative to −17 mV. Analog subtraction of capacity transients was used. B, current-voltage relations are shown for wild type (a), D172H at pH_i 6.3 (b) and D172K (c). C, relationship between chord conductance and membrane potential are shown for wild type (WT, a), D172H at pH_i 6.3 (b) and D172K (c). Chord conductance was computed as $G_K=I_K/(V-E_K)$. Its relation to membrane potential may be fitted by a Boltzmann relation as in Eqn. 1 of the text. For wild type, $V_{1/2}$ = −28.1± −0.5, k=8.8±0.7 (mean±s.e.m, n=5). In this Fig, a linear element, obtained by fitting between currents under large depolarizations (>60 mV) and that at −17 mV, was subtracted. This manoeuvre assumes that K^+ conductance is zero at these positive voltages in all forms of the channel.

functional (Shyng et al, 1997; Minor et al, 1999). However, the substitution in Kir1.1 (ROMK) of the equivalent Asn residue by His does give functional channels (Lu & MacKinnon, 1995). We have found robust currents with Kir2.1 either in its wild type or in any of the following mutant forms: D172H, D172K or D172R. Fig 1A illustrates currents from wild type and from D172H (at pH_i 6.3) and D172K. Each form of the cDNA produces channels showing inward rectification, though this rectification is much weakened by substitution with a basic residue (Fig. 1B, C).

With wild type (Fig. 1Ca), the relationship between K^+ chord conductance and membrane potential could be fit reasonably with a single Boltzmann expression of the form:

$$g'_k = \{1+\exp[(V-V_{1/2})/k]\}^{-1} \quad \ldots\ldots (1),$$

where g'_K is the chord conductance, normalised to its maximum value at negative voltages, $V_{1/2}$ is the voltage at which the normalised conductance is half maximal, and k is a steepness factor. With wild type $V_{1/2}$ = −28.1±0.5 mV and k=8.8±0.7 mV (mean±s.e.m; n=5).

With the mutants (Fig. 1Cb,c), the relationship between chord conductance and voltage could not be easily fit with a Boltzmann relationship. The potassium chord conductance continued to increase as the membrane was hyperpolarised, even down to −180 mV. This apparent lack of saturation appears to be the result of a radically reduced steepness in voltage dependence. The voltage dependence of inward rectifiers is known to depend on $[K^+]_o$, giving an apparent dependence on $(V-E_K)$ (see for example Hodgkin & Horowicz, 1959; Hagiwara & Yoshii, 1980; Leech & Stanfield, 1981), and this property was at least partly conserved in these mutants (Fig. 2). From the reversal potential in different $[K^+]_o$, the mutant channels were highly selective for K^+. With the mutant D172H for a 10-fold change in $[K^+]_o$, reversal potentials changed 56.6 ±0.4 mV (n=6) at pH_i 8.0 and 56.9±0.4 mV (n=6) at pH_i 6.3 (n=6).

Effects of mutation on gating

Associated with inward rectification is an increase in current amplitude with time under hyperpolarisation from E_K (Fig. 1A; Leech & Stanfield, 1981; Ishihara et al, 1989; Stanfield et al, 1994a). This gating process, thought to be associated with the release of polyamines from channels (Fakler et al, 1995; Lopatin et al, 1995), shows voltage dependence and becomes more rapid at more negative voltages. In wild type, the time constants, obtained by fitting the currents with an exponential function, decreased e-fold per 24.5mV and this voltage dependence is independent of pH_i (Fig. 3A).

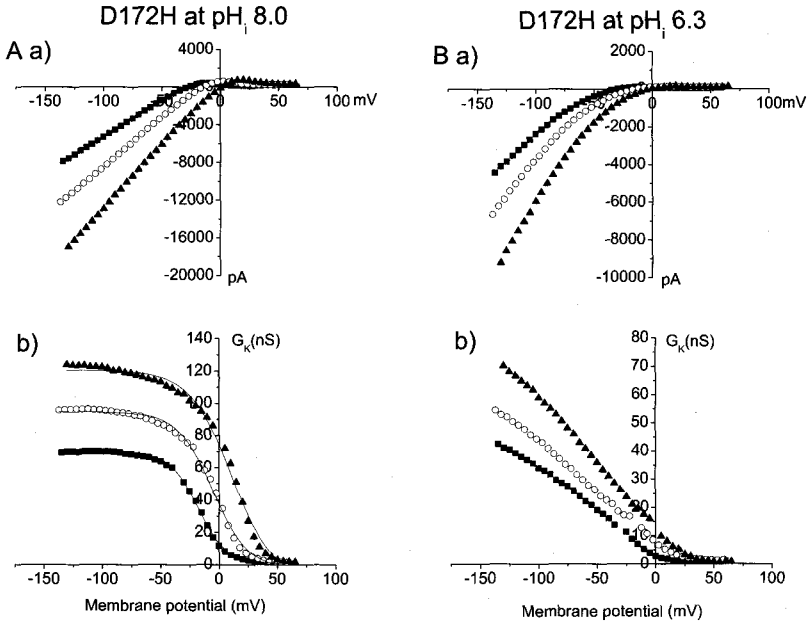

Fig. 2. Dependence of potassium conductance on voltage and $[K^+]_o$. Membrane currents recorded from CHO cells transfected with the D172H mutant of Kir2.1 (pH_i 6.3, A; pH_i 8.0, B). $[K^+]_o$=140 mM (filled triangles), 70 mM (circles) or 35 mM (squares); $[K^+]_i$=140 mM. Voltage pulses were applied in whole cell recording in 5 mV increments from the holding potential set at the calculated potassium equilibrium potential (E_K). Current-voltage relations are shown for D172H at pH_i 8.0 (Aa) and pH_i 6.3 (Ba). The reversal potentials at pH_i 8.0 were 2.2±0.5 (n=6) for 140 mM $[K^+]_o$, −13.6±0.8 (n=6) for 70 mM $[K^+]_o$, and −33.2±0.6 (n=6) for 35 mM $[K^+]_o$. The reversal potentials at pH_i 6.3 were 0.1±0.5 (n=6) for 140 mM $[K^+]_o$, −16.1±0.5 (n=6) for 70 mM $[K^+]_o$, and −34.5±0.3 (n=6) for 35 mM $[K^+]_o$. The relationships between chord conductance and membrane potential are shown for D172H at pH_i 8.0 (Ab) and pH_i 6.3 (Bb). Chord conductance was computed as $G_K = I_K/(V-E_K)$. At pH_i 8.0, the relation of chord conductance to membrane potential may be fitted by a Boltzmann relation using eqn (1) of the text. For 140 mM $[K^+]_o$, $V_{1/2}$ = 8.3±1.2, k = 15.0±0.7 (n=6). Lowering $[K^+]_o$ to 70 mM and 35 mM shifted $V_{1/2}$ to −7.3±0.4 mV with k = 14.9±0.6 (n=6) for 70 mM and $V_{1/2}$ to −17.1±2.8 mV with k = 13.4±1.2 (n=6) for 35 mM. Subtraction of a linear element from the current-voltage relationship was used in fitting Boltzmann relations; this linear element was obtained by fitting between currents under large depolarizations (>60 mV) and that at each E_K. This manoeuvre assumes that K^+ conductance is zero at these positive voltages in all form of the channel.

As expected from the weaker voltage dependence of inward rectification in the mutant forms of the channel, the time constants of activation of currents were also much less dependent on voltage than in wild type (Fig. 3B, C). The time constants were also longer than in wild type in the voltage range over which they were measured.

We fit the relationship between time constant and voltage with the equation

$$\tau(V) = \tau(0).\exp(-V/\kappa) \quad \ldots\ldots\ldots\ldots\ldots (2)$$

where $\tau(V)$ is the voltage dependent time constant, with value $\tau(0)$ at 0mV, and where κ gives the voltage over which $\tau(V)$ changes e-fold. Time constants were reduced e-fold per 461±85 mV (n=6) with D172K and per 501±123 mV (n=11) with D172R (Fig 3B). With D172H, the voltage dependence of gating depended on pH_i, and thus presumably on the protonation of the His side chain. Voltage dependence was steeper if His was largely unprotonated. At pH_i 8.0, the time constant was reduced e-fold per 69±8 mV (n=6). The time constant changed e-fold per 104±12 mV (n=6) at pH_i 7.2, per 253±38 mV (n=6) at pH_i 6.3 and per 525±112 mV (n=4) at pH_i 5.5 (Fig. 3C).

These results suggest either a reduced voltage dependence of polyamine binding when there is a positively charged residue at position 172, perhaps owing to binding to a site closer to the cytoplasmic mouth of the channel, or an intrinsic gating revealed through a reduced polyamine affinity.

Changes in single channel kinetics

The alterations in kinetics seen under whole cell recording will have a correlate in single channel recording. We have recorded single channel currents in inside out patches from wild type and from D172H at pH_i 6.3, applying 60 hyperpolarising pulses from a holding potential of 0 mV to −100 mV under symmetrical, 140 mM $[K^+]$ (Fig. 4). In wild type, channels opened virtually instantaneously in records, though in some (*e.g.* sixth trace in Fig. 4Aa) the channel opened after a long delay. Channel openings were also long lived, interrupted only by brief closures. The ensemble average current was similar to that recorded in whole cell mode (Fig. 4Ab). When the latency to the first opening was measured, the traces where the channel failed to open were not included. The mean latency to first

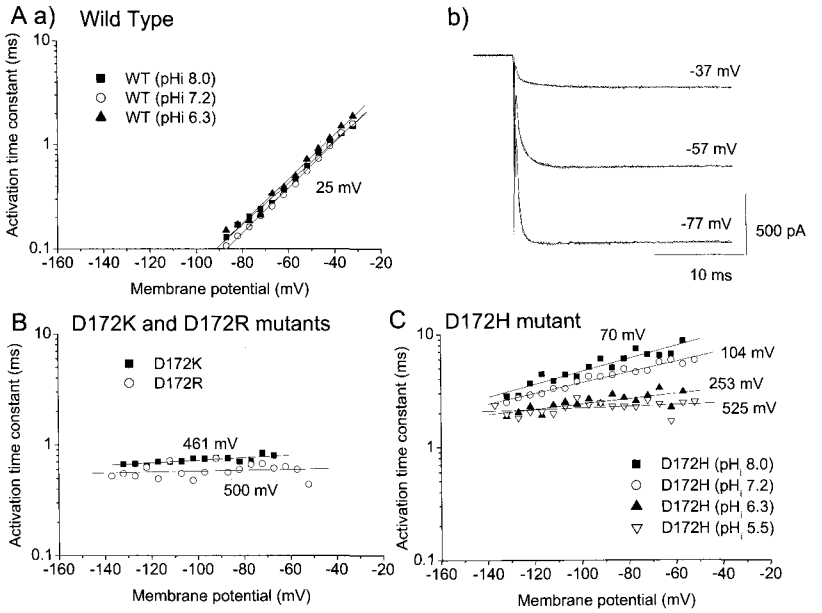

Fig. 3. Gating in wild type and mutant channels. The activation time constants were obtained by fitting the activation phase at each voltage with single exponential function (see Ab). The fits of an exponential function to the inward current are also given as the continuous lines. Capacity transients were subtracted digitally using the transient obtained on stepping back to E_K, where K^+ current is zero. The time constant for activation of inward current (ordinate; log scale) was plotted against membrane potential (abscissa) for wild type (A), D172 K and D172R mutants (B), and D172H mutant at different pH_i (C). The continuous lines show the least-squares fit.

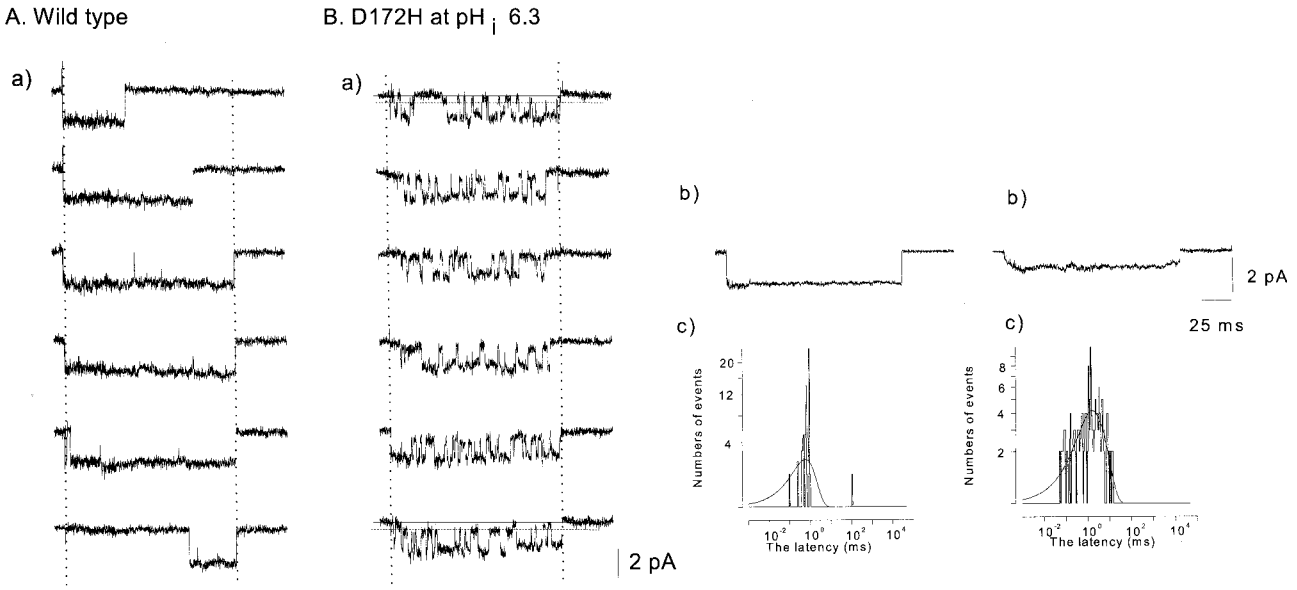

Fig. 4. Ensemble analysis of single channel recordings. A. The single channel currents in WT were activated by a hyperpolarizing pulse to −100 mV from the holding potential of 0 mV in inside-out mode under symmetrical 140 mM $[K^+]_o$. The raw current traces are shown in a). The openings are nearly instantaneous except in one trace among 60. In b), the ensemble average current is shown. The activation was instantaneous. In c), the distribution of the latency to the first opening is shown. The mean time was 0.6 ms. B. The single channel currents in D172H mutant at pH_i 6.3 were activated by a hyperpolarizing pulse to −100 mV from the holding potential of 0 mV in inside-out mode under symmetrical 140 mM $[K^+]_o$. The raw current traces are shown in a). The channels opened later and frequently occupied a substate. In b), the ensemble average current from 60 traces is shown. The current activated exponentially with time constant of 3.4 ms. In c), the distribution of the latency to the first opening is shown. The mean time was 2.4 ms. The vertical dotted lines indicate the start and the end of the 150 ms hyperpolarising pulses.

opening was 0.38±0.10 ms (n=4; Fig. 4Ac).

In D172H, the latency to the first opening increased. As a result, the ensemble average current rises slowly to its maximum value (Fig. 4Bb), with a time constant for the increase of the ensemble average current of 2.4±0.6 ms (n=5), similar to the value recorded in whole cell mode in this voltage range. But other changes in single channel kinetics are also clear from inspection of the records. First, openings are briefer and are interrupted by longer closures. Secondly, there is a prominent substate, whose amplitude is 22.5±1.2% (n=6) of normal at −100 mV (Fig. 4Ba, dashed lines). Often the first opening occurred without this substate (*e.g.* first and sixth records in Fig. 4Ba). We defined an event as the first opening when the event crossed a cursor set at 50% of fully open. The latency to the opening was 1.83±0.27 ms (n=4)(Fig. 4Bc).

In order to study the effect of intracellular pH on the gating in D172H mutant, we recorded unitary currents with 200 mM $[K^+]_o$ and 140 mM $[K^+]_i$. As pH_i was reduced to increase the probability of a positive charge on His, more frequent transitions to substates were seen (Fig. 5). At −100 mV, the number of transitions between fully open and the substate within any opening increased from 0.93±0.12 (n=4) at pH_i 8.0, to 3.8±0.3 (n=4) at pH_i 7.2, and to 16.1±0.5 (n=4) at pH_i 6.3.

The second change was the shape of unitary current-voltage (I-V) relationship (Fig. 5B). The I-V relationship was linear at pH_i 8.0 while that at pH_i 6.3 was not quite linear. This change may be associated with an alteration in the energy profile experienced by permeating cations, when His at position 172 acquires a positive charge. However, the rectification of the unitary current voltage relation is much less than that seen under whole cell recording (e.g. Fig. 1Bb; Fig. 2). The conductances of the open and the substate were 24.5±1.4 pS (n=3) and 7.3±0.3 pS (n=5) at pH_i 8.0, respectively. At pH_i 6.3, the slope conductance in the voltage range −60 mV to −120 mV was 34.1±2.8 pS (n=4; Fig. 5B). There were also changes in dwell times on the open state and close states. The mean open time decreased from 81.1±17.6 ms (n=3) at pH_i 8.0 to 9.1±2.2 ms (n=3) at pH_i 6.3 at −120 mV (measured here in 140 mM $[K^+]_o$; Fig. 5Ca & Da). There was also a decrease in the duration of fast closed times from 2.71±0.33 ms (n=3) at pH_i 8.0 to 1.71±0.10 ms (n=3) at pH_i 6.3. Longer closed times were unchanged in duration (135.0±22.5 ms, n=3, at pH_i 8.0; 98.7±18.7 ms, n=3, at pH_i 6.3; Fig. 5Cb

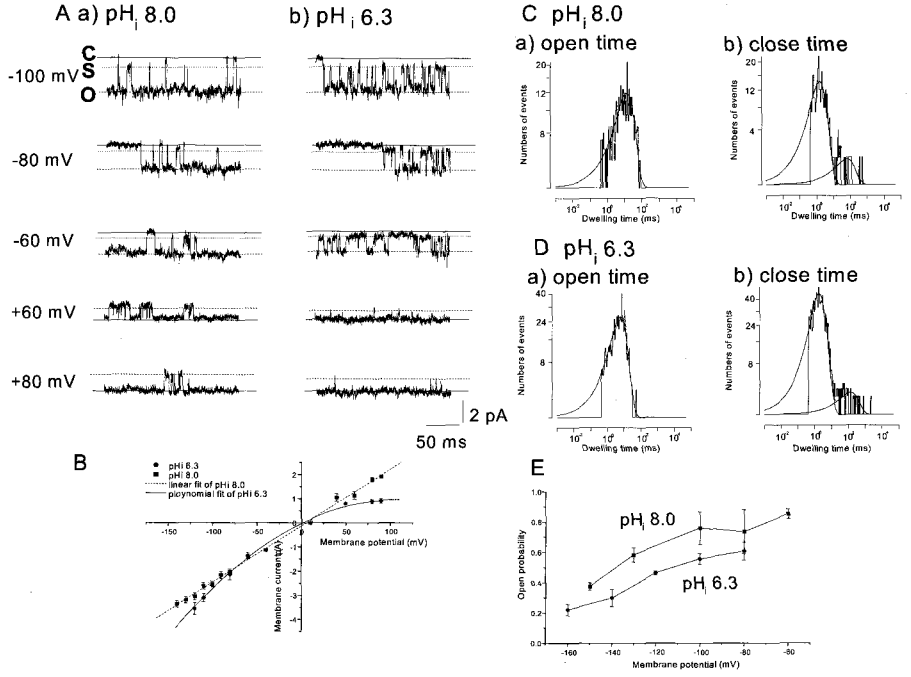

Fig. 5. The single channel currents in D172H mutant. A shows raw current traces at different voltages. Single channel currents were recorded with 140 mM $[K^+]_i$ and 200 mM $[K^+]_o$. *O* denotes the open state, *S* the substate, and *C* the closed state. B shows unitary I/V curves at different pH_i. The amplitudes of single channel currents were obtained using all-point histograms as described in the text. $[K^+]_o$=200 mM, $[K^+]_i$=140 mM. The I/V curve is linear at pH_i 8.0 but not at pH_i 6.3. C shows dwell time distributions in the open and the close states at pH_i 8.0. The single channel currents were recorded at −120 mV with 140 mM $[K^+]_i$ and 140 mM $[K^+]_o$. The open time distribution was fitted to an exponential with τ of 97.5 ms. The close time distribution was fitted to two exponentials with τ_f of 2.97 ms and τ_s of 160.7 ms. D shows the dwell time distributions in the open state and the closed states at pH_i 6.3. The single channel currents were recorded at −120 mV with 140 mM $[K^+]_i$ and 140 mM $[K^+]_o$. The open time distribution was fitted to an exponential with τ of 5.8 ms. The close time distribution was fitted with two exponentials with τ_f of 1.9 ms and τ_s of 130.1 ms. E shows the open state probability plotted against membrane potential. The open state probability decreases as membrane potentials become hyperpolarized. At pH_i 6.3, P_{open} was lower than that at pH_i 8.0 in the range of membrane potentials tested.

& Db). P_{open} decreased as the membrane potential became hyperpolarized as in wild type, but P_{open} at pH_i 6.3 was lower than that in wild type (Fig. 5E; see also So et al, 2001). The kinetics of outward currents was also changed and open times were particularly brief at pH_i 8.0 (Fig. 5Aa, b). Since the unitary current voltage relation shows only weak inward rectification, the brevity and infrequency of openings under depolarisation must contribute to the inward rectification seen under whole cell recording when a positively charged residue is present at position 172.

Effects of mutation on blockage by intracellular polyamines

In preliminary experiments with mammalian cells we used whole cell recording to test the hypothesis that the gating of mutant channels was less dependent on polyamine and was principally intrinsic. In these preliminary experiments using whole cell recording, we buffered polyamines to a lower free concentration by using pipette solutions containing ATP at 10 mM (see *e.g.* Fakler et al, 1995). Such a procedure resulted in a shift in the voltage dependence of inward rectification in wild type channels to more positive voltages, consistent with the reduction in free polyamine concentration. Such a shift was not seen with D172H at pH_i 6.3, even over a period of 40 min, consistent with a reduced dependence on polyamines.

To test the hypothesis of intrinsic gating more directly, we expressed channels in *Xenopus* oocytes and used macropatch recording. Here, we could directly alter spermine concentrations in ways that we have found difficult to achieve in mammalian (MEL or CHO) cells. Fig. 6 shows records for currents from D172H, first in cell attached mode (Fig. 6A) and then with the macropatch excised into polyamine-free intracellular solution.

After excision and with pH_i 9.0, inward rectification is very weak in the absence of intracellular polyamine, though outward currents at the most positive voltages show some decline with time during depolarisation (Fig. 6E). As pH_i was reduced, so increasing the protonation of His in position 172, inward rectification became more obvious as did the time-dependent change of current during voltage steps, suggestive of channel gating. A time-dependent increase in current under hyperpolarisation became particularly prominent, once pH_i was reduced to 7.2 (Fig. 5C). Acidification reduced the amplitude of steady state outward currents (Fig. 5F). The apparent pK_a was 7.43± 0.05 (n=3) at +40 mV. The pK_a was voltage dependent and the voltage dependence was calculated from dissociation constant for the protonation reaction according to the expression:

$$K_d\ (V)=K_d(0).\exp(-z\,\delta VF/RT) \quad \text{.....(3)}$$

The values found for the effective valency ($z\,\delta$) gave a mean of 0.51±0.06 (n=3).

Our results suggest that an intrinsic gating had been induced by the presence of a positively charged residue at

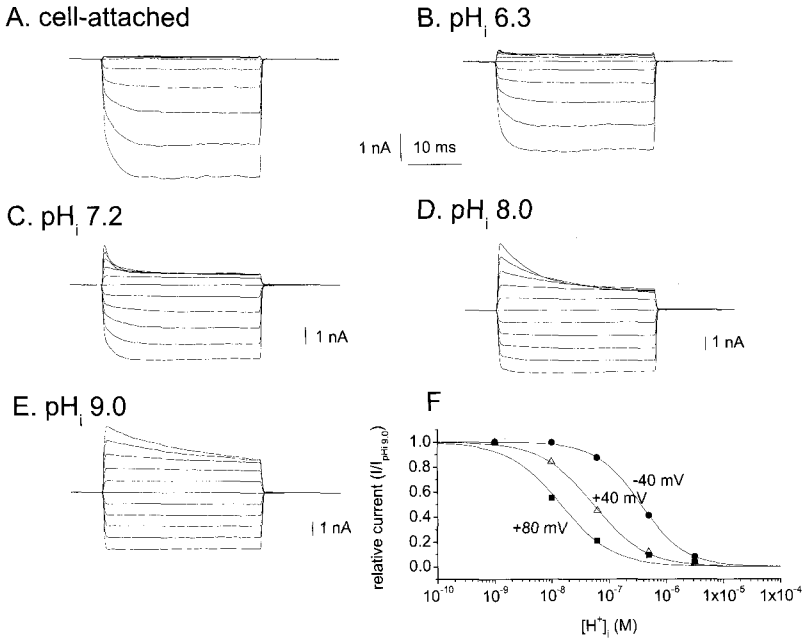

Fig. 6. Membrane currents recorded in inside-out patches from *Xenopus* oocytes in the D172H mutant. Macropatch currents were recorded as follows. (A) cell-attached mode. (B) pH_i 6.3, excised inside-out macropatch. (C) pH_i 7.2; (D) pH_i 8.0; (E) pH_i 9.0, all in inside-out patches. Voltage pulses were applied in 10 mV increments from 100 mV to −100 mV from a holding potential of 0 mV, equivalent to potassium equilibrium potential (E_K). F, Hill plots against proton concentration of the amplitudes of the currents recorded at the end of the voltage pulse normalised to the value found at pH_i 9.0 ($I/I_{pHi\ 9.0}$). The curves superimposed on the data correspond to least-squares fits to the equation: $I/I_{pHi\ 9.0} = 1/[1+([H^+]_i/K_D)^n]$.

position 172. To test whether this process was due to the use of HEPES buffer (see Guo & Lu, 2000), we carried out experiments with phosphate buffer and found essentially the same results. Here, the apparent pK_a was 7.20±0.08 (n=3) at 40 mV; the effective valency ($z\delta$) was 0.44±0.05 (n=3).

Affinity for polyamines – altered affinity and voltage dependence

We next asked whether polyamines have any influence on inward rectification in these mutant channels. We used ramp changes of membrane potential (from −100 to +100 mV over 1s) to generate current voltage relations under conditions where spermine was added at concentrations from 1 nM to 1 mM (Fig. 7, 8).

First we describe the results with wild type channels (Fig. 7). The current voltage relation was reasonably linear over the voltage range −100 to +50 mV after 20 min washout of endogenous polyamine but was not linear beyond +50 mV, probably owing to a very low residual polyamine concentration (<<1 nM). We measured the K_d for spermine by plotting the fractional current against the spermine concentration in which it was measured. At +50 mV, the K_d was 6.3±3.3 nM (n=3) at pH_i 8.0 and 5.8±1.9 nM (n=3) at pH_i 6.3. These values are close to that of 5.9 nM found by Lee et al. (1999) for wild type channels at +50 mV (see also Yang et al, 1995; Taglialatela et al, 1995). The voltage dependence was calculated from the expression:

$$K_d(V)=K_d(0).\exp(-z\,\delta\,VF/RF) \quad \ldots\ldots\ldots\ldots (3)$$

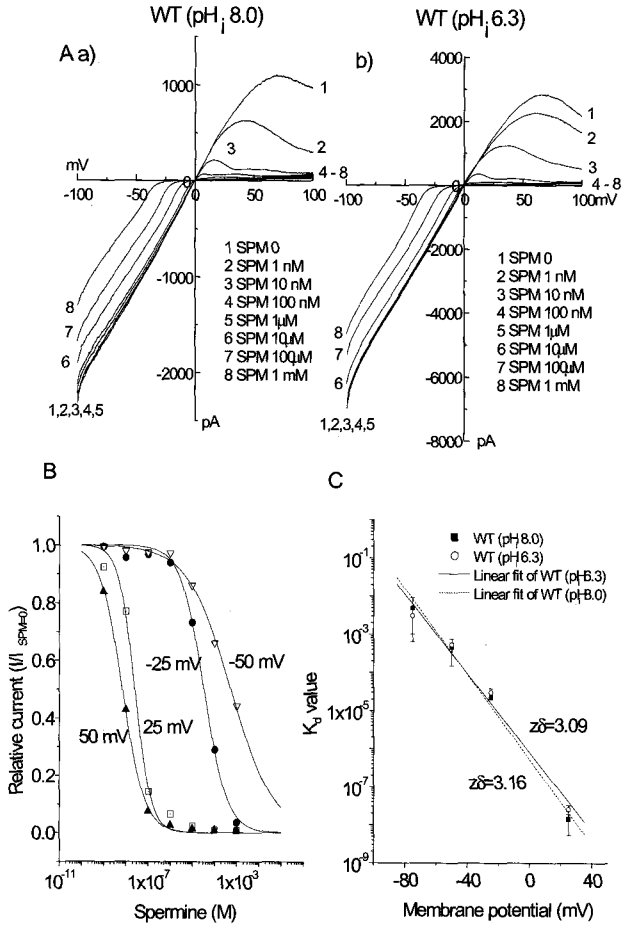

Fig. 7. The effect of spermine in wild type. Currents were recorded from excised inside out macropatches from *Xenopus* oocytes expressing wild type channels. A stimulating ramp pulse was applied from −100 mV to 100 mV for 1 s from a holding potential of 0 mV. (A) at pH_i 8.0; (B) at pH_i 6.3. (C) shows Hill plots against spermine concentration of the current ($I/I_{SpM=0}$) obtained in the presence of intracellular spermine, normalised to that found in the absence of spermine at pH_i 6.3 at −50 mV, −25 mV, 25 mV and 50 mV. The curves superimposed on the data correspond to least-squares fits to the equation: $I/I_{SpM=0} = 1/[1+([H^+]_i/K_D)^n]$. (D) K_D (ordinate, logarithmic scale) is plotted against membrane potential (abscissa). The line superimposed on the data is a least-squares fit to $K_D(V)= K_D(0)$ exp $(-z\,\delta FV/RT)$.

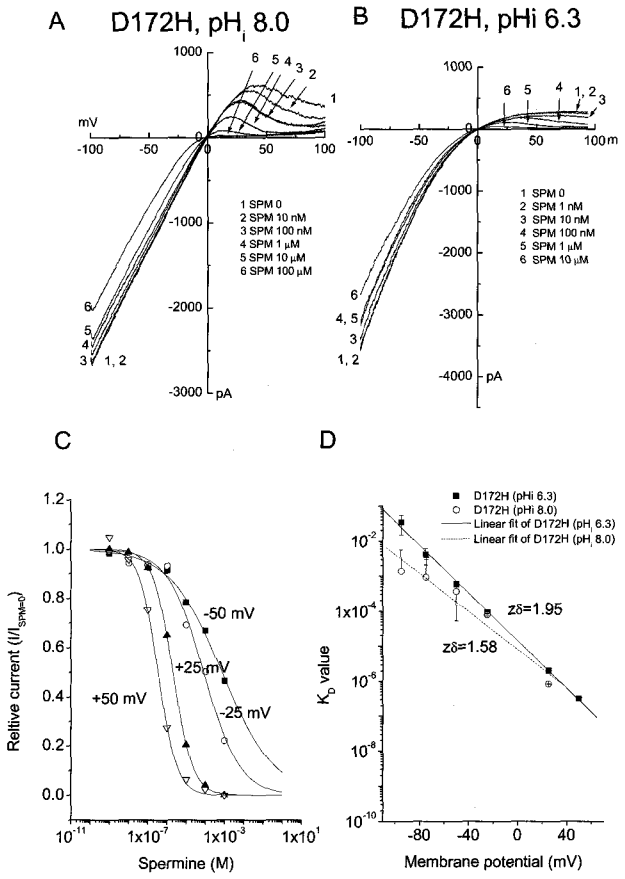

Fig. 8. The effect of spermine in D172H mutant. Currents were recorded from excised inside out macropatches from *Xenopus* oocytes expressing D172H channels. A stimulating ramp pulse was applied from −100 mV to 100 mV for 1 s from a holding potential of 0 mV. (A) at pH_i 8.0; (B) at pH_i 6.3. (C) shows Hill plots for D172H at pH_i 6.3 at −50 mV, −25 mV, 25 mV and 50 mV. The curves superimposed on the data correspond to least-squares fits to the equation: $I/I_{SpM=0} = 1/[1+([H^+]_i/K_D)^n]$. (D) K_D (ordinate, logarithmic scale) is plotted against membrane potential (abscissa). The line superimposed on the data is a least-squares fit to $K_D(V)= K_D(0)$ exp $(-z\,\delta FV/RT)$.

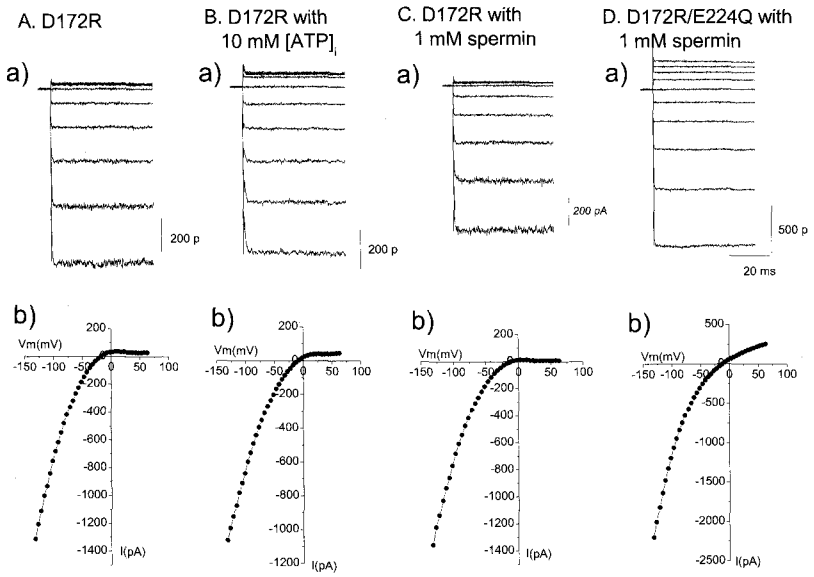

Fig. 9. Buffering or raising intracellular spermine. A-Da), membrane currents recorded from CHO cells transfected with mutant forms of Kir2.1. $[K^+]_o$=70 mM; $[K^+]_i$=140 mM. Voltage pulses were applied from 63 mV to −132 mV in 5 mV increments from a holding potential of −17 mV, equivalent to potassium equilibrium potential (E_K). Currents were illustrated in response to 20 mV steps up to 80 mV positive and down to 100 mV negative to the holding potential. Analog subtraction of capacity transients was used. A-Db), current-voltage relations are shown for Ab) D172R without 10 mM ATP_i, Bb) D172R with 10 mM ATP_i, Cb) D172R with 1 mM spermine and Db) D172R/E224Q with 1 mM spermine.

The values found for the effective valency ($z\,\delta$) were 3.16±0.10 (n=3) and 3.09±0.07 (n=3) at pH_i 8.0 and 6.3, respectively (Fig. 7D).

Polyamine affinity was reduced with the mutation D172H and the reduction was greater at pH_i 6.3 (Fig. 8). At +50 mV, the K_d was 54±13 nM (n = 3) at pH_i 8.0 and 325±37 nM (n = 4) at pH_i 6.3. The value found at pH_i 8.0 is similar to that found for the mutant D172N by Lee et al. (1999; see also Yang et al, 1995; Taglialatela et al, 1995).

We hypothesised that, when the residue at position 172 carries a positive charge, spermine would move less far into the channel, but this hypothesis may not be correct. It appears that spermine may move less far into the channel, whether His at position 172 is protonated or not. The effective valency was altered from that in wild type at both pH_i 8.0 and 6.3, with $z\,\delta$ = 1.58±0.07 (n=3) and 1.95±0.09 (n=4) at pH_i 8.0 and 6.3 respectively (Fig. 8D). Since the mutations E224G and E299G are known to have a much greater effect on polyamine affinity (Yang et al, 1995; Taglialatela et al, 1995; Lee et al, 1999; Kubo & Murata, 2001), these C-terminus glutamate residues may be the more important in regulating high affinity binding of spermine.

Mutations of E224 and double mutants

To investigate further the involvement of one of these C-terminal residues, E224, we changed the residue at this position and also made double mutants, replacing both D172 and E224. Mutants of E224 were: E224H, E224K and E224R. Double mutants were: D172H/E224G, D172H/E224Q; D172K/E224G, D172K/E224Q; D172R/E224G; and

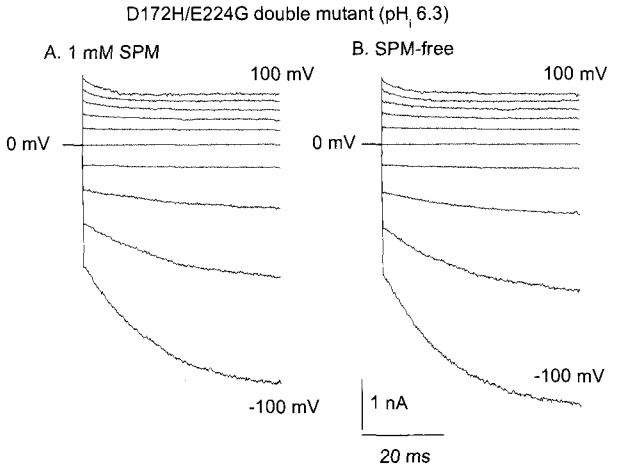

Fig. 10. The effects of 1 mM spermine on current in a double mutant, D172H/E224G at pH_i 6.3. Membrane currents recorded from *Xenopus oocyte* injected with mutant D172H/E224G cRNA. Recordings are at pH_i 6.3 in the presence of 1 mM spermine (A) and in the absence of spermine (B). $[K^+]_o$=140 mM; $[K^+]_i$=140 mM. Currents were recorded from a macropatch excised in the inside out configuration. Voltage pulses were applied in 5 mV increments from 100 mV to −100 mV from a holding potential of 0 mV. Currents illustrated were in response to 20 mV steps to 100 mV positive and down to 100 mV negative to the holding potential. Analog subtraction of capacity transients was used. Spermine (1 mM) was without effect on the outward currents in double mutants.

D172R/E224Q. All of these channels showed inward rectification. We used two strategies to investigate in the CHO cell expression system whether polyamines are involved in gating. In the first (Fig. 9), we introduced a high concentration of ATP (10 mM) in the patch pipette; in the second we introduced spermine in the patch pipette at 1 mM. In mutants of D172 alone (*e.g.* D172R), ATP increased outward current owing to its binding of polyamine, indicating that these outward currents are reduced in part by polyamine blockage (Fig. 9B). Adding 1 mM spermine to the patch pipette blocked outward currents in D172R (Fig. 9C), but outward currents could still be recorded under these conditions with the double mutant D172R/E224G (Fig. 9D). Similar results were recorded with D172H (pH_i 6.3)/E224G and D172K/E224G.

Again, to test directly whether gating occurs completely independently of polyamine in the double mutants, we recorded currents in macroscopic patches from oocytes in the absence and presence of 1mM spermine (Fig. 10). One mM spermine failed to inhibit currents in the D172H/E224G double mutant at pH_i 6.3, though channels showed inward rectification, gating with changes of voltage in a way that must now be completely intrinsic.

DISCUSSION

The open state probability of strong inward rectifiers depends both on voltage and on $[K^+]_o$, the voltage dependence of P_{open} changing with the quantity $\frac{RT}{F}.\ln [K^+]_0$ (Hagiwara & Yoshii, 1979; Leech & Stanfield, 1981). Thus, raising extracellular K^+ opens strong inward rectifier potassium channels, such as Kir2.1. Not only do these channels set negative resting potentials, but their activation by K^+ also permits them to play more complex but highly important physiological roles. K^+ activation of Kir2.1 underlies its central role in the vasodilatation that can occur in response to a raised $[K^+]_o$, which hyperpolarises vascular smooth muscle in certain regions of the circulation (*e.g.*, see Zaristsky et al, 2000). Such activation may also permit channels to act as conduits for the clearance of K^+ from restricted areas of the extracellular space (*e.g.* Stanfield et al, 2002). Examples are the T-systems of cardiac and skeletal muscle (Wallinga et al, 1999; Leonoudakis et al, 2001) and from around node of Ranvier (Mi et al, 1996).

This phenomenon of dependence on both voltage and $[K^+]_o$ is generally interpreted in simple terms, where an open ion channel is blocked by intracellular polyamines, whose occupancy is reduced through competition with extracellular K^+ (Ficker et al, 1994; Lopatin et al, 1994; Fakler et al, 1995; Lopatin & Nichols, 1996). Thus, channels are more likely to be blocked at depolarised voltages, but less likely to be blocked if $[K^+]_o$ is raised. It is not fully understood how the voltage dependence of channel open state probability is linked so precisely to the change in $\frac{RT}{F}.\ln [K^+]_0$ but mutations in the selectivity filter suggest a central role for an Arg residue at the outer mouth of the pore (*e.g.* Kubo, 1996). A difficulty with the hypothesis is that the inner mouth of the channel has a large diameter (Lu et al, 1999) and may be incompletely blocked by a single polyamine molecule. This fact has led some authors to suggest the simultaneous binding of more than one polyamine molecule (Yang et al, 1995; Xie et al, 2002), though this suggestion seems unlikely if molecules repel each other electrostatically as may be expected.

Many channels of the inward rectifier family clearly undergo conformation changes associated with channels moving between shut and open states. Thus, G-protein coupled channels of the Kir3.0 subfamily require interaction with $G\beta\gamma$ to be open (Yi et al, 2001; Sadja et al, 2001). K_{ATP} channels of the Kir6.0 subfamily are shut when they bind ATP (Drain et al, 1998; Loussouarn et al, 2000). Channels of the Kir1.0 subfamily are gated by interactions both with extracellular K^+, which opens channels, and intracellular H^+, which shuts them (*e.g.* Schulte et al, 2001). Mutations of the selectivity filter or pore helix affect the activation of channels by K^+; mutations of intracellular protein domains affect the response to protonation (Schulte et al, 2001). Some of these gating changes are likely to be akin to those occurring in KcsA (Doyle et al, 1998; Perozo et al, 1999) Kv (Liu et al, 1997) and K(Ca) channels (Jiang et al, 2002a) through the flexion of the inner pore helix about a conserved helix-breaking glycine residue (Jiang et al, 2002a, b). With the exception of Kir4.0 and Kir5.0, this glycine residue is conserved throughout the Kir channel family.

Analysis of the effects of certain mutations of Kir2.0 suggests that these channels may also undergo conformation changes. Thus, mutations of the pore region show changes in single channel kinetics without detectable alterations in the phenomenon of inward rectification (or of selectivity; So et al, 2001). These changes may be associated with changes of the conformation of the selectivity filter itself. Related to this, involvement of the selectivity filter appears to be the role of external K^+in stabilising channels in an open state (Shieh, 2000). This stability is lost if foreign permeant cations (Tl^+, NH_4^+, Rb^+) carry current and channels inactivate under hyperpolarisation (Ashcroft & Stanfield, 1983; Shieh & Lee, 2001). These descriptions are strongly supportive of the hypothesis that Kir2.0 channels do possess intrinsic gating processes and are not permanently open structures, subject only to blockage by extrinsic factors. The conformation changes here are likely to involve the selectivity filter and may be akin to C-type inactivation in Kv channels (*e.g.* Lopez-Barneo et al, 1993).

The experiments of this paper show that certain mutations of the inner, less selective part of the pore can also result in channel gating. The residues altered are those thought to act as part of the receptor for polyamines. Changes in these residues affect the phenomenon of inward rectification itself. The principal changes made in the present experiments are where basic residues replace the crucial aspartate (D172) of the second transmembrane domain (M2). In all cases (D172H at low pH_i, D172K, D172R), channels show inward rectification that is a shallow function of membrane potential. This inward rectification occurs under conditions, where the affinity for polyamine is much reduced, though not abolished. However, inward rectification persists in the absence of intracellular polyamines, and is weakened only slightly in such absence.

The unitary current voltage relation itself now shows some inward rectification, presumably owing to the introduction of an energy barrier to ionic transfer with the introduction of positive charges in the permeation pathway (see for example Yang et al, 1995). But this inward

rectification is weak compared with that shown under whole cell recording. Evidently, the channels open and shut and have a reduced P_{open} at positive voltages. This gating process is not associated with blockage by the buffer used in the experiment, since essentially the same results are obtained whether HEPES or phosphate buffer is used (see Guo & Lu, 2000). It seems likely that the low P_{open} around E_K of mutants with a basic residue replacing D172 accounts for the failure of such mutants to provide a K^+ uptake pathway in K^+ transport deficient yeast, as used in the mutation experiments of Minor et al. (1999).

Additional mutation of a C-terminal Glu (E224) renders affinity for spermine undetectable. Channels continue to open and shut, but now behave in an identical fashion whether spermine is present or not.

Thus, it seems likely to us that Kir2.1 channel can undergo conformation changes, that these become obvious when a positive charge is introduced at position 172, and that these conformation changes generate inward rectification. One possibility is that these positive charges, introduced through mutation, imperfectly mimic the introduction of positive charges with the binding of spermine in the pore. The conformation changes found in the mutants generate inward rectification, though the rectification is shallower than that seen in wild type channels in the presence of polyamines. A reasonable hypothesis is that the interaction of Kir2.0 channels with polyamines permits a conformation change to occur in a way that sets the normal physiological behaviour of the ion channels.

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