The role of a single aspartate residue in ionic selectivity and block of a murine inward rectifier K^+ channel Kir2.1

C. J. Abrams, N. W. Davies, P. A. Shelton and P. R. Stanfield*

Ion Channel Group, Department of Cell Physiology and Pharmacology and Centre for Mechanisms of Human Toxicity, University of Leicester, PO Box 138, Leicester LE1 9HN, UK

1. The effects of Rb^+ and Cs^+ as blocking ions were investigated on wild-type and mutant forms of the inward rectifier K^+ channel, IRK1 (Kir2.1).

2. In wild-type channels, Rb^+ blockage was voltage dependent, increasing and then falling with increasing hyperpolarization.

3. Rb^+ blockage was abolished by replacing Asp172 in the M2 domain of the pore-forming subunit by Asn, but was re-established by a change to Gln, narrowing the pore. Blocking affinity was reduced in D172Q, and was also reduced by replacing Gly168 in M2 by Ala.

4. Cs^+ blockage was also abolished in D172N but was re-established in D172Q.

5. There appears to be a balance between charge and pore size in determining whether ions block or permeate. A major part of the selectivity of Kir2.1 is associated with Asp172 in the M2 domain.

The resting potentials of many cell types are determined by members of a family of 'inward rectifier' potassium channels (Kir; Doupnik, Davidson & Lester, 1995). Such channels allow little K^+ efflux under depolarization owing to voltage-dependent blockage by intracellular polyamines and Mg^{2+} (Matsuda, Saigusa & Irisawa, 1987; Stanfield *et al.* 1994*a*; Fakler *et al.* 1994; Lopatin, Makhina & Nichols, 1994; Ficker, Taglialatela, Wible, Henley & Brown, 1994). Many members of the Kir family are more selective than members of the voltage-gated potassium (Kv) channel family in allowing only K^+ among the alkali metal ions to permeate readily; Rb^+ permeates Kv channels but blocks certain inward rectifier channels (Adrian, 1964). In this paper we show that a negatively charged residue (Asp172 in Kir2.1), essential for channel gating (Fakler *et al.* 1994; Stanfield *et al.* 1994*b*; Wible, Taglialatela, Ficker & Brown, 1994) because it confers a major part of the binding site for polyamines, also confers the strong selectivity. The residue lies in one (M2) of two membrane-spanning domains and outside the domain H5, usually thought to contribute the most selective part of the pore (Pongs, 1993). Mutation of Asp172 to Asn (D172N) abolishes blockage by Rb^+, giving high permeance of Rb^+, and radically alters blockage by Cs^+. However mutation to Gln (D172Q), narrowing the pore, re-establishes both Rb^+ and Cs^+ blockage.

METHODS

Molecular biology

Wild-type and mutant DNA were prepared as described in a previous paper (Sta0nfield *et al.* 1994*b*). One additional mutation (G168A; see Fig. 1*A*) was generated using a modification of the method of Kunkel (1985). Firstly, single-stranded template DNA containing uracils was produced. This template was rescued from the *ung*− *Escherichia coli* strain, RZ1302, harbouring the pBluescript SK−/IRK1. Secondly, a G168A sense oligonucleotide was 5′-phosphorylated and annealed to the template so that wild-type and/or mutant double-stranded DNA hybrids could be generated. Finally, the double-stranded DNA hybrid was transformed into the competent *ung*+ *E. coli* strain JM109. Plasmid DNA was purified from ampicillin-resistant colonies and mutants were verified by automated sequencing. Wild-type and mutant Kir2.1 sequences were expressed in murine erythroleukaemia (MEL) cells for study by whole-cell recording (Shelton *et al.* 1993).

Electrophysiology

Membrane currents were measured using an Axopatch 200 or 200A amplifier (Axon Instruments), filtered at 2–5 kHz (−3 dB, 8-pole Bessel), digitized at 10 kHz using a Labmaster TL-1 interface (Axon Instruments) and analysed on a 80486-based microcomputer. Patch pipettes were filled with a solution containing (mM): Hepes, 10; EGTA, 10; $MgCl_2$, 1·18 (calculated free $[Mg^{2+}]$, 1·0); pH 7·2 (adjusted with KOH); KCl to bring $[K^+]$ to 140 mM. External solutions contained (mM): Hepes, 10; KCl (or RbCl), 70; NaCl or *N*-methyl-D-glucamine, 70; $CaCl_2$, 2; $MgCl_2$, 2; pH 7·2 with NaOH (70 mM NaCl) or HCl (70 mM *N*-methyl-D-glucamine). CsCl was

* To whom correspondence should be addressed.

This manuscript was accepted as a Short Paper for rapid publication.

added to the external solution at the concentrations given in the text; RbCl was substituted for part of the *N*-methyl-D-glucamine. A solution-changing system enabled cells to be exposed to control and test solutions in turn. Experiments were carried out at room temperature, *ca* 20 °C. Results are given as means ± S.E.M.

RESULTS

Cs^+ and Rb^+ block Kir2.1 in a voltage-dependent manner

Both Cs^+ and Rb^+ block native inward rectifier potassium channels in a steeply voltage-dependent manner (Gay & Stanfield, 1977; Standen & Stanfield, 1980). Figure 1 shows the same features in cloned inward rectifier channels. Blockage by Cs^+ increased as the membrane potential was made more negative (Fig. 1*B* and *D*), blockage taking a few milliseconds to reach steady state. The blockage of native channels was fitted assuming one-to-one interaction between Cs^+ and the channel (Hagiwara, Miyazaki & Rosenthal, 1976), according to:

$$Cs^+ + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} CsR, \quad (1)$$

where k_{+1} is the association rate constant and k_{-1} is the dissociation rate constant. The equilibrium dissociation

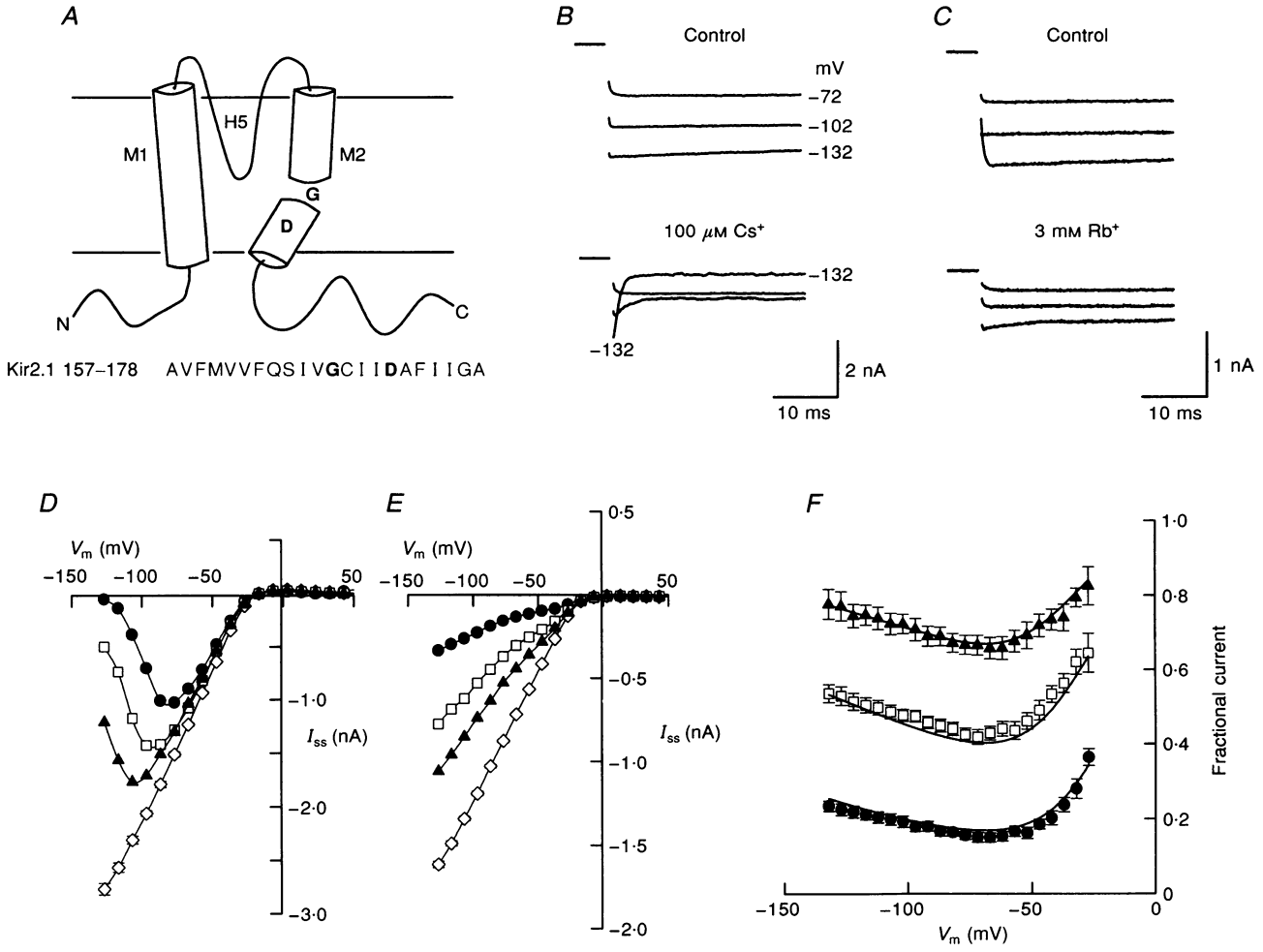

Figure 1. Blockage of Kir2.1 by Cs^+ and Rb^+

A, diagram of the proposed structure of the pore-forming subunit, showing membrane-spanning domains M1 and M2 and the location of G168 and D172. *B* and *C*, membrane currents carried by K^+ in the absence (top; Control) and presence (bottom) of blocking Cs^+ (*B*) and Rb^+ (*C*). The holding potential (V_h) was set to the theoretical equilibrium potential for potassium (−17 mV) and the membrane was hyperpolarized to the voltages indicated. *D*, current–voltage relations in control (◇), 30 (▲), 100 (□) and 300 μM Cs^+_o (●). I_{ss} (ordinate) denotes steady-state current, and V_m, membrane potential. *E*, current–voltage relations in control (◇), 1 (▲), 3 (□) and 10 mM Rb^+_o (●). *F*, fractional current remaining after Rb^+ blockage in 1 (▲), 3 (□) and 10 mM Rb^+_o (●). Lines show the best fits to eqn (4), with $A = 22{\cdot}6$ mM; $B = 0{\cdot}82$ mM; $x = 1{\cdot}57$; $y = 0{\cdot}27$.

constant, K_D, of this reaction will then be voltage dependent (see Fig. 4), falling with hyperpolarization as follows:

$$K_D(V) = K_D(0)\exp\left(\frac{\delta' VF}{RT}\right). \quad (2)$$

$K_D(V)$ is the voltage-dependent equilibrium constant, whose value is $K_D(0)$ at 0 mV; V is the membrane potential; and R, T and F have their usual thermodynamic meanings. δ', expected to give the fraction of the electrical field through which Cs^+ moves to its blocking site, is 1·57 in the case of the present experiments (Fig. 4). The value >1 for δ' is consistent with a multi-ion pore: permeant K^+ must also be moved to make way for the blocking Cs^+ (see for example Hille & Schwarz, 1978).

Blockage by Rb^+ also initially increased with hyperpolarization, but was relieved at yet more negative voltages as Rb^+ moved through to the intracellular solution (Fig. 1*C*, *E* and *F*). Rb^+ blockage may be fitted with a semi-empirical expression related to that used to describe Cs^+ blockage. We assume that Rb^+ can move from the extracellular solution to

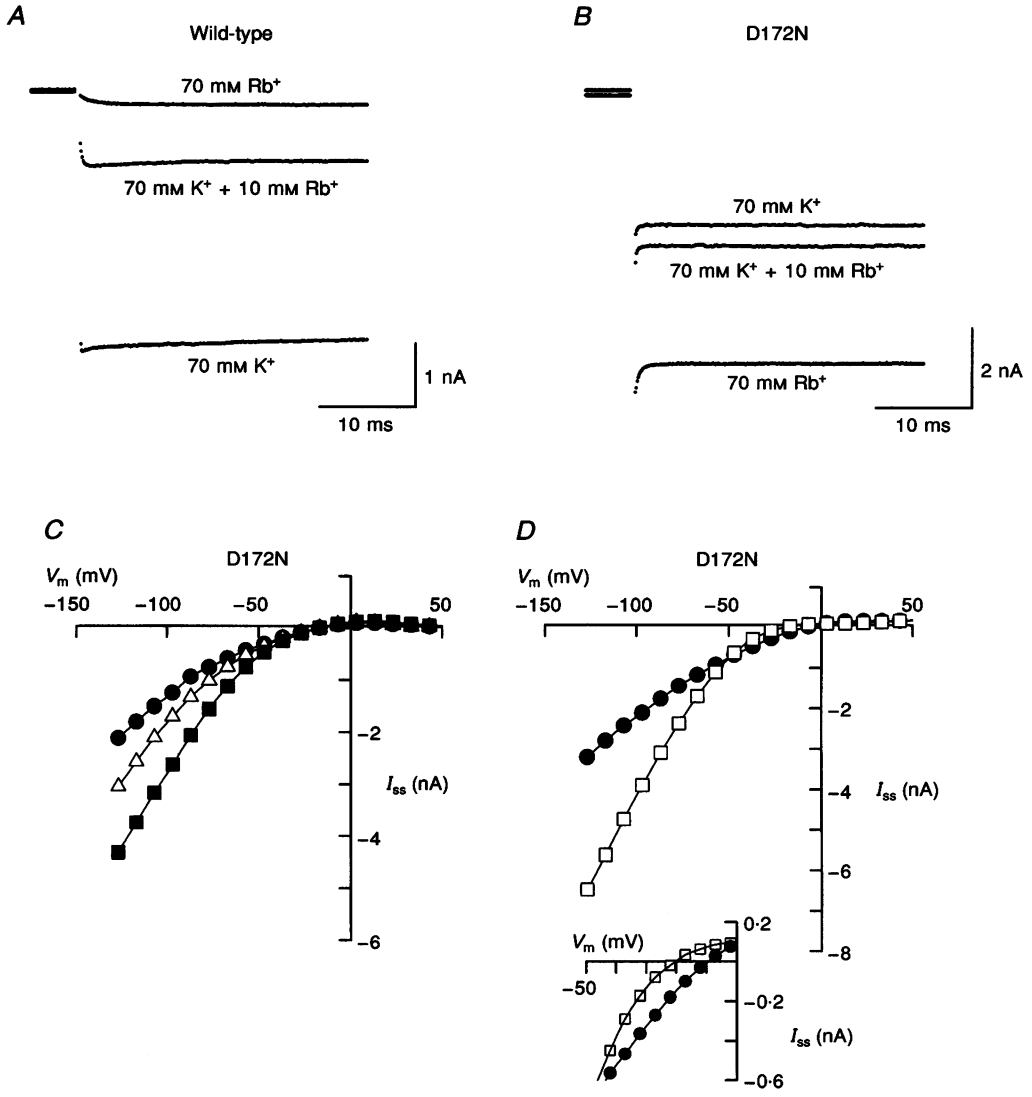

Figure 2. Rb^+ currents in wild-type and D172N

A, wild-type currents measured after hyperpolarization to −132 mV (V_h, −17 mV). The extracellular solution contained either 70 mM K^+, 70 mM Rb^+ or 70 mM K^+ + 10 mM Rb^+ as indicated. *B*, currents recorded from the D172N mutant under the same conditions as *A*. *C*, current–voltage relations for D172N in control (●), 10 (△) and 30 mM Rb_o^+ (■). *D*, current–voltage relations for D172N with 70 mM K_o^+ (●) and 70 mM Rb_o^+ (□). Inset, expanded axes showing more detail.

its blocking site and then on to the intracellular solution (Standen & Stanfield, 1980). Thus:

$$\underset{Outside}{Rb^+} + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} RbR \xrightarrow{k_{-2}} \underset{Inside}{Rb^+} + R. \quad (3)$$

k_{-1} and k_{-2} are dissociation rate constants for Rb^+ leaving to the extracellular and intracellular solutions, respectively.

The fractional current (f) in the presence of Rb^+ may then be given by:

$$f = \frac{A\exp(xVF/RT) + B\exp(-yVF/RT)}{[Rb]_o + A\exp(xVF/RT) + B\exp(-yVF/RT)}, \quad (4)$$

where $A \times \exp(xVF/RT)$ represents k_{-1}/k_{+1} and is akin to $K_D(V)$ in eqn (2) (x being equivalent to δ') while $B \times \exp(-yVF/RT)$ represents k_{-2}/k_{+1} (y being the algebraic sum of the fractions of the voltage field associated with k_{-2} and k_{+1}; see Standen & Stanfield, 1980). Figure 1*F* shows fits of eqn (4) to the relationship between fractional current in the presence of Rb^+ and voltage, with x set at 1·57 (the value found for δ' with Cs^+ blockage, see Fig. 4*E*), $y = 0{\cdot}27$, $A = 22{\cdot}6$ mM, and $B = 0{\cdot}82$ mM.

Blockage occurs at D172

Strong inward rectifier channels possess a negatively charged residue in M2 (Fig. 1*A*; Doupnik *et al.* 1995). In Kir2.1, this residue is Asp172 (Kubo, Baldwin, Jan & Jan, 1993). Mutation of the residue to Asn (D172N) permits larger outward K^+ currents (Stanfield *et al.* 1994*b*; Wible *et al.* 1994): it also radically alters channel blockage by Rb^+ (Fig. 2) and by Cs^+ (Fig. 4).

Adding Rb^+ to a 70 mM K_o^+ solution now resulted in an increase in current and no sign of blockage (Fig. 2*B* and *C*). This result suggests that Rb^+ has become a permeant cation as a result of the mutation, a suggestion confirmed by the experiment of replacing K^+ in the external solution by Rb^+ (Fig. 2*B* and *D*). Over a range of negative membrane potentials, currents carried by Rb^+ (I_{Rb}) are much larger than those carried by K^+ (I_K). For example, at −117 mV, $I_{Rb}/I_K = 0{\cdot}09 \pm 0{\cdot}02$ ($n = 7$) in wild-type channels, but is

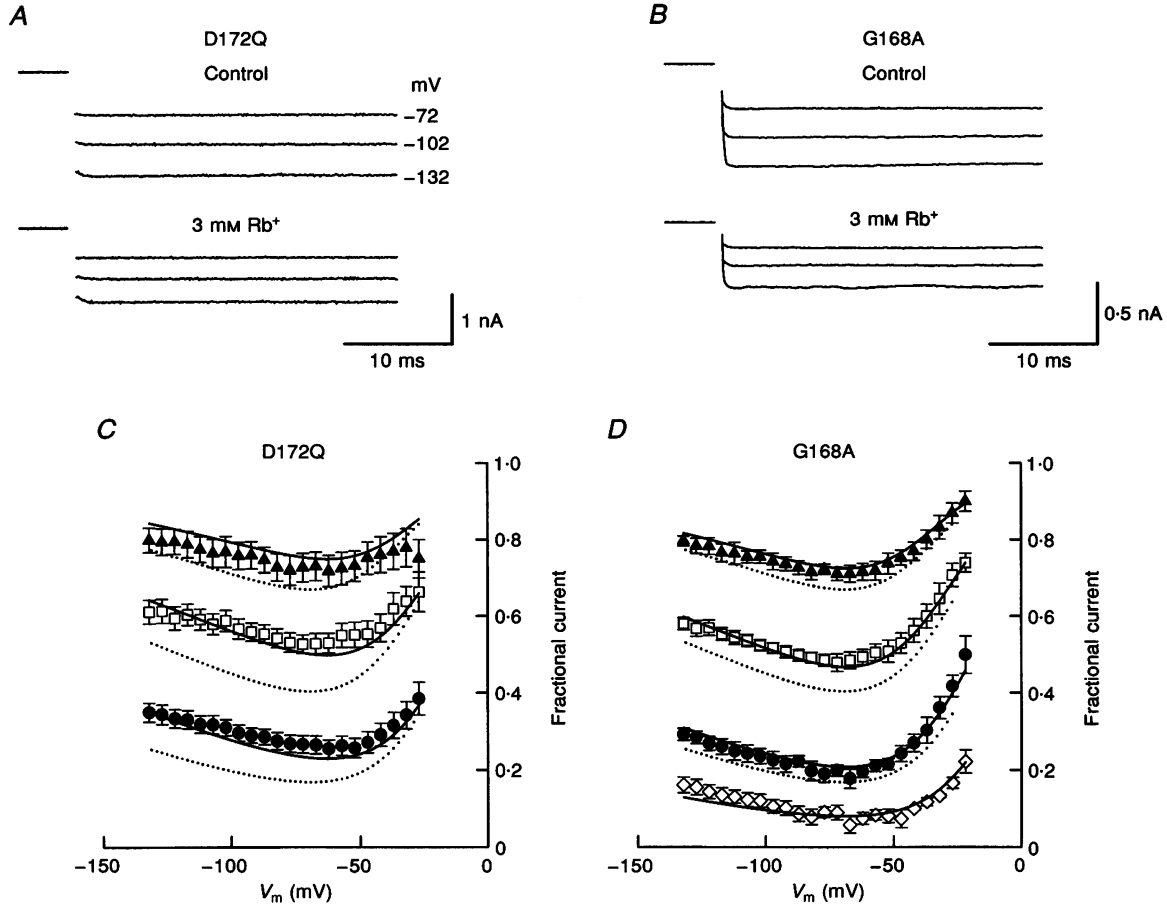

Figure 3. Rb^+ block of D172Q and G168A

Currents in control and in the presence of 3 mM Rb^+ in D172Q (*A*) and G168A (*B*) at the voltages indicated (V_h, −17 mV). *C*, D172Q; fractional current remaining in 1 (▲), 3 (□) and 10 mM Rb_o^+ (●). Lines show the best fits to eqn (4), with $A = 22{\cdot}3$ mM; $B = 1{\cdot}29$ mM; $x = 1{\cdot}57$; $y = 0{\cdot}27$. *D*, G168A; fractional current remaining in 1 (▲), 3 (□), 10 (●) and 30 mM Rb^+ (◇). Lines show the best fits to eqn (4), with $A = 28{\cdot}4$ mM; $B = 1{\cdot}07$ mM; $x = 1{\cdot}57$; $y = 0{\cdot}27$. Dotted lines show fits to wild-type for comparison.

2·05 ± 0·28 ($n = 4$) in D172N. Thus Rb^+, previously the less permeant ion because of its binding in the channel, now traverses the channel more quickly than does K^+.

We have calculated the permeability ratio (P_{Rb}/P_K) from the change of reversal potential occurring when Rb^+ is substituted for external K^+ (Hille, 1992). With wild-type, the reversal potential (E_{rev}) changed by −12·3 ± 4·8 mV ($n = 7$), with $P_{Rb}/P_K = 0·68 \pm 0·11$. With D172N, it changed by −13·1 ± 2·8 mV ($n = 4$), with $P_{Rb}/P_K = 0·61 \pm 0·06$, not significantly altered from wild-type. The permeability ratio is similar to that for native inward rectifier channels of frog skeletal muscle ($\Delta E_{rev} = -10·3$ mV; $P_{Rb}/P_K = 0·66$; Standen & Stanfield, 1980). In the present experiments, the ratio may be expected to be determined by the H5 domain believed to line the outer part of the pore. Recent experiments (Reuveny, Jan & Jan, 1996) have also shown that P_{Rb}/P_K is unaltered in D172N if external K^+ is replaced by Rb^+.

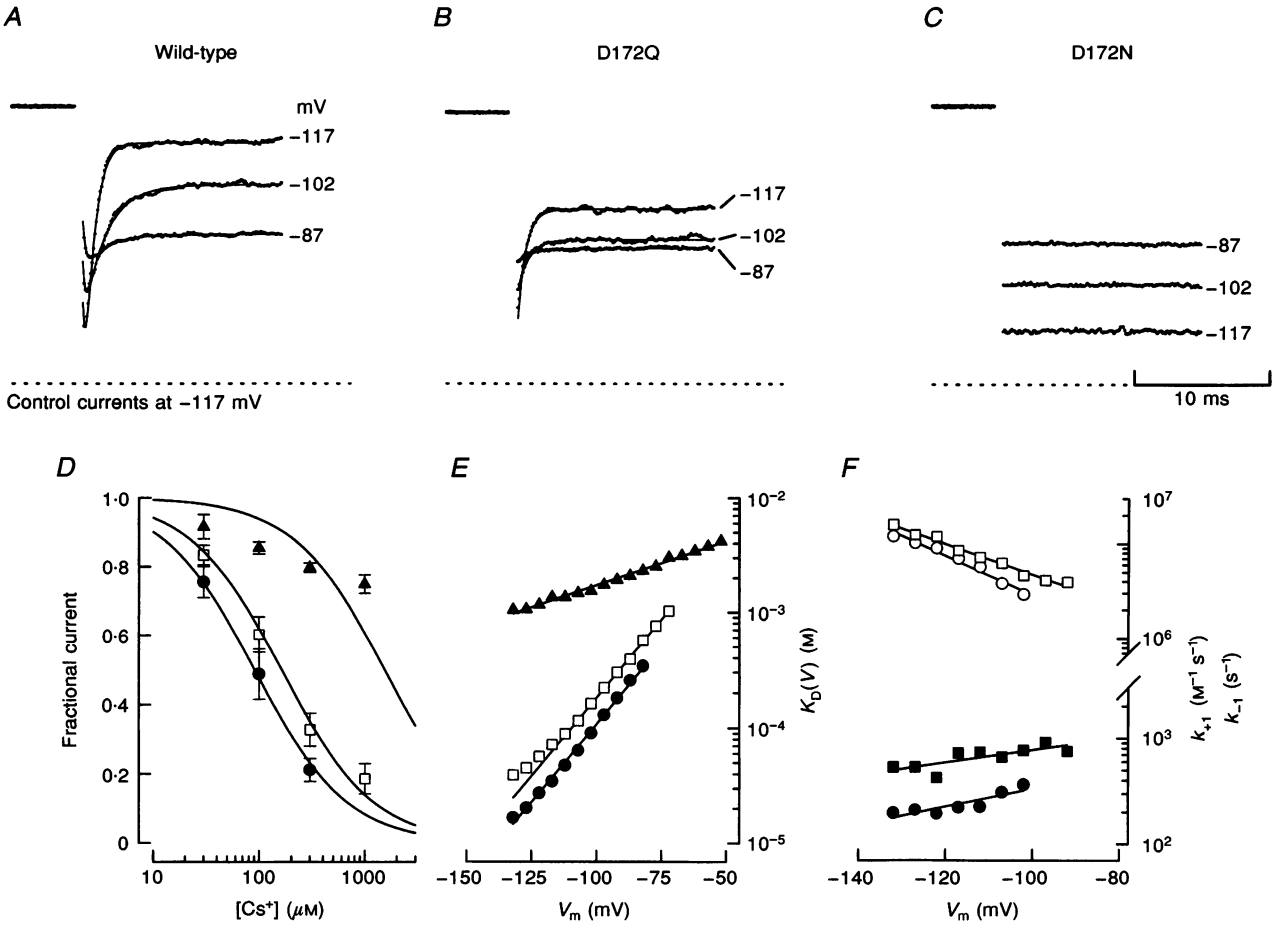

Figure 4. Cs^+ block in wild-type, D172Q and D172N

A–C, currents in wild-type, D172Q and D172N with 300 μM Cs_o^+ at the voltages indicated. Currents normalized to those measured without external Cs^+ in each cell at −117 mV (dashed lines, $A = -4·2$, $B = -3·7$ and $C = -1·3$ nA). V_h, −17 mV. In *A* and *B*, fits of the block are superimposed. *A*, the time constant of the block (τ_b) was isolated from that of gating (τ_a) by fitting the current to: $f(t) = [c + a(1 - \exp(-t/\tau_a))] \times [1 - b(1 - \exp(-t/\tau_b))]$, where c and a are the amplitudes of the instantaneous and time-dependent components and b the fractional block. *B*, time dependence of block fitted to a single exponential, as gating is abolished in D172Q. Values of τ_b at −87, −102 and −117 mV were 1·52, 1·42 and 0·61 ms in wild-type and 1·15, 0·82 and 0·61 ms in D172Q, respectively. *C*, Cs^+ block of D172N; note lack of time dependence and weaker voltage dependence of the block. *D*, concentration dependence of Cs^+ blockage at −102 mV in wild-type (●), D172Q (□) and D172N (▲). Lines show the best fit to the Hill–Langmuir equation, with Hill coefficient = 1, and $K_D = 91$, 162 and 1540 μM for wild-type, D172Q and D172N, respectively. *E*, voltage dependence of K_D for wild-type (●), D172Q (□) and D172N (▲). Lines are fits to eqn (2) giving $K_D(0)$ and δ' values, respectively, of 54·0 mM and 1·57 for wild-type, 90·8 mM and 1·57 for D172Q and 10·3 mM and 0·45 for D172N. *F*, plot of k_{+1} (open) and k_{-1} (closed symbols) against membrane potential for wild-type (circles) and D172Q (squares). k_{+1} values were obtained from τ_b by fitting regression lines to $1/\tau_b = k_{+1}[Cs^+] + k_{-1}$; k_{-1} values were obtained using K_D and k_{+1}.

Narrowing the pore restores blockage

In order to check whether structural changes expected to alter pore diameter would affect blockage, we investigated other mutations. Mutation D172Q, where the longer side-chains of Gln residues might narrow the pore by up to 0·31 nm (equivalent to two $-CH_2-$ groups; one from each Gln on opposing sides of the pore), restored Rb^+ blockage (Fig. 3) and also block by Cs^+ (Fig. 4). Thus, narrowing the pore compensates in large measure for the removal of the negative charge at this site.

However, detailed analysis of the blockage by Rb^+ indicated a reduced block at all voltages compared with wild-type channels, so that the compensation is not complete. Fitting the results with eqn (4), with x and y fixed at the values used for wild-type, gives $A = 22{\cdot}3$ mM (unaltered from wild-type) and $B = 1{\cdot}29$ mM, increased from its wild-type value, indicating that Rb^+ is less well trapped at its blocking site.

Effects of attempts to widen the pore

We have also attempted to investigate effects of a mutation which is expected to widen the pore. The M2 domain is thought to have an α-helical structure. It possesses a helix-breaking Gly residue (conserved in most but not all sub-families of Kir; Doupnik *et al.* 1995) which may be expected to flex the M2 helix. Preliminary modelling proposed that this flexion brings the Asp residues (D172) in the four subunits forming the pore closer to each other (Stanfield *et al.* 1994*b*). We have therefore investigated effects of replacing the residue by the helix-forming Ala. This mutant, G168A, produced functional channels (Fig. 3*B*), indicating that a flexion in M2 is not essential for channel function. However the currents activated more rapidly under hyperpolarization. This increase in the rate of activation was approximately fourfold suggesting that channels release polyamines more rapidly. These channels were also blocked by Rb^+, so the widening of the channel is insufficient to remove this blockage (Fig. 3*D*). However the results show small reductions in the block, consistent with the mutation making a small increase in pore diameter, so reducing the affinity for Rb^+. Fitting the results to eqn (4) gives increased values for A of 28·4 and B of 1·07 mM from the values found in wild-type.

Cs^+ blockage also occurs at D172

Experiments on native channels suggested that Rb^+ and Cs^+ blockage could occur at the same site (Standen & Stanfield, 1980) and this supposition is confirmed by our experiments with mutant channels. The blockage by Cs^+ is further analysed in Fig. 4. In the presence of Cs^+, currents inactivated along an exponential time course in wild-type channels and in D172Q (Fig. 4*A* and *B*). The inactivation occurred more rapidly in D172Q. The affinity for Cs^+ was also lower in D172Q ($K_D(0) = 91$ mM) than in wild-type ($K_D(0) = 54$ mM; Fig. 4*D* and *E*), but showed an identical voltage dependence, with $\delta' = 1{\cdot}57$ (see eqn (2)). These changes in affinity and rate of inactivation are associated with a change in the dissociation rate constant (k_{-1}; Fig. 4*F*; see eqn (1)). The association rate constant (k_{+1}), in which the majority of the voltage dependence resides, is virtually unaffected by the mutation. Since Cs^+ must move through unaltered parts of the pore to reach its blocking site, it might be expected that k_{+1} would be little changed by the mutation. In the case of D172Q, $K_D(V)$ is larger than expected from eqn (2) at the most negative voltages (Fig. 4*E*), indicating that at these voltages Cs^+ may be able to move more easily through to the intracellular fluid.

Mutation D172N abolished the steeply voltage-dependent blockage by Cs^+, removing also the inactivation of currents associated with this blockage (Fig. 4*C*). However, an additional Cs^+ blocking site is revealed in this mutant, apparently nearer the external mouth of the pore (Fig. 4*D*). Fitting the blockage using eqn (2) now gives $K_D(0) = 10{\cdot}3$ mM and $\delta' = 0{\cdot}45$. This additional blocking site, which may account for the reduction in instantaneous currents seen in wild-type and D172Q (Fig. 4*A* and *B*), may lie in the unaltered H5 region.

DISCUSSION

Strong inward rectifier channels, whose properties allow them to support a number of physiological functions (Hille, 1992), pass little outward current because they are blocked by intracellular Mg^{2+} and polyamines (Matsuda *et al.* 1987; Fakler *et al.* 1994; Ficker *et al.* 1994; Lopatin *et al.* 1994). One of the major residues involved in polyamine blockage is an Asp residue towards the inner part of the pore in the M2 transmembrane domain (Fakler *et al.* 1994; Stanfield *et al.* 1994*b*; Wible *et al.* 1994). Hand-in-hand with the property of strong inward rectification is strong selectivity against Rb^+, and we here show that the same Asp residues (D172) confer this selectivity.

It is the H5 region of potassium (and other voltage-gated) channels that is normally regarded as the 'selectivity filter'. In inward rectifier channels, this region must still be strongly selective, since it has been shown that H^+ cannot get through H5 to the position equivalent to D172 in ROMK1 (Kir1.1), though intracellular H^+ can reach the position (Lu & MacKinnon, 1995). However, it appears that both Rb^+ and Cs^+ are able to permeate H5 in Kir2.1, blocking strongly only when they reach D172. Selection against Rb^+ then occurs because the cation becomes trapped by a ring of negative charges. But the effects of removal of the negative charge can be compensated for by lengthening the side chain of the amino acid residue at this site, indicating a balance between charge and pore diameter in deciding ionic blockage or permeance.

ADRIAN, R. H. (1964). The rubidium and potassium permeability of frog muscle membrane. *Journal of Physiology* **175**, 134–159.

DOUPNIK, C. A., DAVIDSON, N. & LESTER, H. A. (1995). The inward rectifier potassium channel family. *Current Opinion in Neurobiology* **5**, 268–277.

FAKLER, B., BRANDLE, U., BOND, C. H., GLOWATZKI, E., KONIG, C., ADELMAN, J. P., ZENNER, H. P. & RUPPERSBERG, J. P. (1994). A structural determinant of differential sensitivity of cloned inward rectifier K^+ channels to intracellular spermine. *FEBS Letters* **356**, 199–203.

FICKER, E., TAGLIALATELA, M., WIBLE, B. A., HENLEY, C. M. & BROWN, A. M. (1994). Spermine and spermidine as gating molecules for inward rectifier K^+ channels. *Science* **266**, 1068–1072.

GAY, L. A. & STANFIELD, P. R. (1977). Cs^+ causes a voltage-dependent block of inward K currents in resting skeletal muscle fibres. *Nature* **267**, 169–170.

HAGIWARA, S., MIYAZAKI, S. & ROSENTHAL, N. P. (1976). Potassium current and the effect of cesium on this current during anomalous rectification of the egg cell membrane of a starfish. *Journal of General Physiology* **67**, 621–638.

HILLE, B. & SCHWARZ, W. (1978). Potassium channels as multi-ion single-file pores. *Journal of General Physiology* **72**, 409–442.

HILLE, B. (1992). *Ionic Channels of Excitable Membranes*, 2nd edn. Sinauer Associates Inc, Sunderland, MA, USA.

KUBO, Y., BALDWIN, T. J., JAN, Y. N. & JAN, L. Y. (1993). Primary structure and functional expression of a mouse inward rectifier potassium channel. *Nature* **362**, 127–133.

KUNKEL, T. A. (1985). Rapid and efficient site-directed mutagenesis without phenotype selection. *Proceedings of the National Academy of Sciences of the USA* **82**, 488–492.

LOPATIN, A. N., MAKHINA, E. N. & NICHOLS, C. G. (1994). Potassium channel block by cytoplasmic polyamines as the mechanism of intrinsic rectification. *Nature* **372**, 366–369.

LU, Z. & MACKINNON, R. (1995). Probing a potassium channel pore with an engineered protonatable site. *Biochemistry* **34**, 13133–13138.

MATSUDA, H., SAIGUSA, A. & IRISAWA, H. (1987). Ohmic conductance through the inward rectifier K channel and blocking by internal Mg^{2+}. *Nature* **325**, 156–159.

PONGS, O. (1993). Structure–function studies on the pore of potassium channels. *Journal of Membrane Biology* **136**, 1–8.

REUVENY, E., JAN, Y. N. & JAN, L. Y. (1996). Contributions of a negatively charged residue in the hydrophobic domain of the IRK1 inward rectifier K^+ channel to K^+-selective permeation. *Biophysical Journal* **70**, 754–761.

SHELTON, P. A., DAVIES, N. W., ANTONIOU, M., GROSVELD, F., NEEDHAM, M., HOLLIS, M., BRAMMAR, W. J. & CONLEY, E. C. (1993). Regulated expression of K^+ channel genes in electrically silent mammalian cells by linkage to β-globin gene-activation elements. *Receptors and Channels* **1**, 25–37.

STANDEN, N. B. & STANFIELD, P. R. (1980). Rubidium block and rubidium permeability of the inward rectifier of frog skeletal muscle fibres. *Journal of Physiology* **304**, 415–435.

STANFIELD, P. R., DAVIES, N. W., SHELTON, P. A., KHAN, I. A., BRAMMAR, W. J., STANDEN, N. B. & CONLEY, E. C. (1994*a*). The intrinsic gating of inward rectifier K^+ channels expressed from the murine IRK1 gene depends on voltage, K^+ and Mg^{2+}. *Journal of Physiology* **475**, 1–7.

STANFIELD, P. R., DAVIES, N. W., SHELTON, P. A., SUTCLIFFE, M. J., KHAN, I. A., BRAMMAR, W. J. & CONLEY, E. C. (1994*b*). A single aspartate residue is involved in both intrinsic gating and blockage by Mg^{2+} of the inward rectifier, IRK1. *Journal of Physiology* **478**, 1–6.

WIBLE, B. A., TAGLIALATELA, M., FICKER, E. & BROWN, A. M. (1994). Gating of inward rectifier K^+ channels localized to a single negatively charged residue. *Nature* **371**, 246–249.

Acknowledgements

We thank The Wellcome Trust, the MRC and the Royal Society for support. C.J.A. is a Wellcome Trust Prize Student; N.W.D. is a Royal Society University Research Fellow. We thank Dr Mike Sutcliffe for suggesting the mutation G168A.

Author's email address

P. R. Stanfield: prs@le.ac.uk

Received 29 February 1996; accepted 4 April 1996.