The intrinsic gating of inward rectifier K^+ channels expressed from the murine IRK1 gene depends on voltage, K^+ and Mg^{2+}

P. R. Stanfield*†, N. W. Davies*†, P. A. Shelton†‡, I. A. Khan*, W. J. Brammar†‡, N. B. Standen*† and E. C. Conley†‡

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

1. We describe the cloning of the inward rectifier K^+ channel IRK1 from genomic DNA of mouse; the gene is intronless.
2. The IRK1 gene can be stably expressed in murine erythroleukaemia (MEL) cells. Such transfected cells show inward rectification under whole-cell recording.
3. Channels encoded by the IRK1 gene have an intrinsic gating that depends on voltage and $[K^+]_o$. Rate constants are reduced e-fold as the driving force on K^+ ($V - E_K$) is reduced by 24·1 mV.
4. Removal of intracellular Mg^{2+} permits brief outward currents under depolarization. The instantaneous current–voltage relation may be fitted by an appropriate constant field expression.
5. Removal of intracellular Mg^{2+} speeds channel closure at positive voltages. In nominally zero $[Mg^{2+}]_i$, rate constants for the opening and closing of channels, processes which are first order, are similar to those of native channels.

Recently a new family of potassium channels has been described (Ho *et al.* 1993; Kubo, Baldwin, Jan & Jan, 1993*a*; Kubo, Reuveny, Slesinger, Jan & Jan, 1993*b*), which includes certain channels that are sensitive to intracellular ATP (ROMK1: Ho *et al.* 1993) and channels showing inward rectification (IRK1: Kubo *et al.* 1993*a*; GIRK1: Kubo *et al.* 1993*b*). The family is characterized by proteins predicted to possess only two membrane spanning α-helices (M1 and M2: Ho *et al.* 1993; Kubo *et al.* 1993*a*), rather than the six (S1–S6) of voltage-gated K^+ channels. In particular, inward rectifiers lack the voltage-sensing S4 domain with its high incidence of basic amino acids (e.g. Pongs, 1992).

Although the phenomenon of inward rectification depends in part on a voltage-dependent blockage of channels by intracellular cations such as Mg^{2+} (Matsuda, Saigusa & Irisawa, 1987; Vandenberg, 1987), native inward rectifiers also show intrinsic gating. This gating involves channel open probability (P_o) increasing under hyperpolarization, a process that is reversed at positive voltages (Hagiwara & Yoshii, 1979; Leech & Stanfield, 1981). But the gating also depends on the concentration of permeant ion, apparently depending on the difference between voltage and the equilibrium potential for potassium ($V - E_K$) (Hagiwara & Yoshii, 1979; Leech & Stanfield, 1981; Ishihara, Mitsuiye, Noma & Takano, 1989; Pennefather, Oliva & Mulrine, 1992).

In this paper, we describe experiments in which we have isolated the IRK1 gene from mouse genomic DNA. Using the polymerase chain reaction (PCR) and complete sequencing of the amplified products, we show that the IRK1 gene lacks introns in the protein coding region. We also show that IRK1, expressed in our experiments in a murine cell line, possesses the intrinsic voltage- and $[K^+]_o$-dependent gating described for native inward rectifier. In addition, as has been described in heart muscle, the ability of the channel to pass outward current and its gating are strongly affected by intracellular Mg^{2+} (Matsuda *et al.* 1987; Ishihara *et al.* 1989). Absence of intracellular Mg^{2+} permits channels to pass outward current when open, but speeds gating so that channels close very quickly at positive voltages.

METHODS

Molecular biological methods and cell culture

Genomic DNA was extracted from the liver of a mouse (killed by cervical dislocation) for analysis and subsequent amplification of the IRK1 gene by PCR. To maintain fidelity and product yield of PCR, it was necessary to determine empirically final concentrations of Mg^{2+} (1–10 mM), deoxynucleoside triphosphates (dNTP, 0·1–0·2 mM) and *Taq* polymerase (1·0–5·0 units per 100 μl reaction). All reactions used 20 pmol of sense and antisense primers and 50 ng genomic DNA template, prepared according to the methods of Sambrook, Fritsch & Maniatis (1989). Amplification conditions were initially one cycle of 95 °C for 5 min, 55 °C for 5 min, 72 °C for 2 min, followed by a final 72 °C extension step for 10 min, holding at 4 °C. Primer annealing specificity was also adjusted empirically: non-specific bands were reduced by raising primer annealing and extension temperatures. In order to prevent spurious priming during routine DNA amplifications, the

'touchdown' PCR procedure of Don, Cox, Wainwright, Baker & Mattick (1991) was also used.

Amplification products were resolved on a 3% NuSieve agarose gel, and the 1306 base pair (b.p.) band, encompassing the entire IRK1 coding region, was recovered using the Geneclean™ (Bio 101 Inc., La Jolla, CA, USA) procedure and was then subcloned into *Sma*I-cleaved and dephosphorylated pUC18 plasmid cloning vector using conventional methods. Several IRK1–pUC18 recombinant clones were recovered and verified as being undeleted at the termini of the insert by restriction mapping.

Flanking *Eco*RI and *Hind*III sites in the cloning vector polylinker were used to transfer the IRK1 coding region (from the IRK1–pUC18 clone with the IRK1 sequence 5′ to the *Eco*RI site in pUC18) into the plasmid vector pBluescript SK$^+$ (Stratagene Inc., La Jolla, CA, USA). An *Eco*RI/*Xho*I fragment from this IRK1–pBluescript SK$^+$ recombinant was then re-excised and ligated to the β-globin LCR-promoter vector pEV3 to produce a functional expression construct. The IRK1/pEV3 expression construct was transfected into log phase murine erythroleukaemia (MEL) cells by electroporation. This method and the subsequent induction of expression prior to analysis were as previously described (Shelton *et al.* 1993).

Electrophysiological methods

Whole-cell recording was used to measure membrane currents, using an Axopatch 200A amplifier. Large outside-out membrane patches, which reduced both capacity transients and series resistance errors, were used to investigate channel gating properties in a few experiments. Currents were filtered at 5 kHz (−3 dB, 8-pole Bessel), digitized at 10 or 20 kHz using a Labmaster TL-1 interface (Axon Instruments, Foster City, CA, USA), and analysed on a 486 computer. Patch pipettes were pulled from 1·5 mm o.d. capillaries, fire-polished, and filled with solutions containing (mM): Hepes, 10; EGTA, 10; $MgCl_2$, 1·18 or 10 (calculated free Mg^{2+} 1·0 and 8·6 mM); KOH to pH 7·2; KCl to bring $[K^+]$ to 140. In Mg^{2+}-free solution EDTA replaced EGTA. Glucose was added to bring the osmolality to 280 mosmol kg^{-1}. Extracellular solutions contained (mM): KCl, 35 or 70; NaCl, 105 or 70; $CaCl_2$, 2; $MgCl_2$, 2; and Hepes buffer, (pH 7·2) 10.

Analog means were used to correct capacity transients as far as possible. In some instances, capacity transients were subtracted digitally, either using the transient associated with stepping the voltage back to E_K (scaled if necessary) or, in the presence of internal Mg^{2+}, where outward K^+ currents are

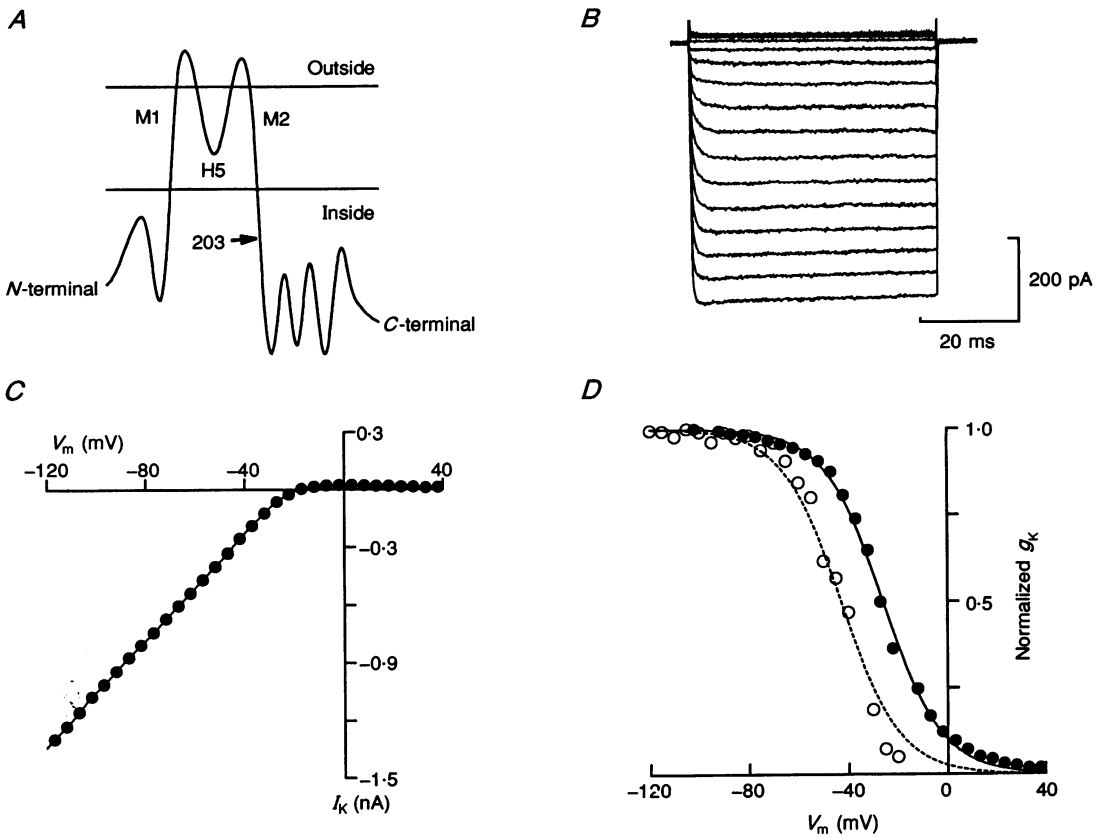

Figure 1. The IRK1 gene encodes inward rectifier channels

A, illustration of the membrane-spanning domains of the channel peptide as described by Kubo *et al.* (1993*a*). The arrow indicates the position of residue 203, where there was a conservative amino acid substitution from the sequence of Kubo *et al.* (1993*a*; see text). *B*, membrane currents obtained with 70 mM $[K^+]_o$ under depolarizations in 5 mV steps up to 70 mV from E_K, and under hyperpolarizations in 5 mV steps down to 60 mV from E_K. *C*, current–voltage relation given by the currents in *B*. V_m, membrane potential; I_K, potassium current. *D*, relationship between chord conductance (g_K) and membrane potential for records in *B* and *C* (●) and for a cell in 35 mM $[K^+]_o$ (○). A Boltzmann curve was fitted to ● and shifted 17·5 mV negative to fit ○.

negligible under large depolarizations, by scaling and subtracting the current during a 70 mV depolarization. Leakage subtraction was carried out by scaling appropriately and then subtracting any holding current at E_K. A least-squares method was used to fit rates of activation and of deactivation of currents.

Electrophysiological experiments were carried out at room temperature, 20–23 °C.

RESULTS

The protein coding region of the murine IRK1 gene does not contain introns

In order to determine whether the protein coding regions of the chromosomal copy of the IRK1 channel sequence contained introns, three sense and three antisense primers were synthesized, based on the cDNA sequence of Kubo *et al.* (1993*a*; see Fig. 1*A*). Primers were chosen to cover the entire 1284 b.p. open reading frame of the IRK1 gene product, including the Kozak initiation ribosome binding sequence (Kozak, 1984) and thirteen nucleotides beyond the thymine of the terminator codon TGA (base 1282). Relative to the adenine of the initator ATG (position +1), the sense primers hybridized to bases −8 to +10, +445 to +462, and +853 to +870 and the antisense primers to bases +445 to +462, +853 to +870, and +1281 to +1298. Following pair-wise amplification of the genomic DNA template, three overlapping fragments were produced, as expected if the IRK1 genomic sequence was co-linear with the IRK1 cDNA. Thus −8 to +10 sense and +445 to +462 antisense gave a 470 b.p. product and the other primer pairs gave 425 and 445 b.p. products, respectively. The murine IRK1 gene therefore contains no introns.

Stable expression of the murine IRK1 gene

The primer pair capable of amplifying the entire intronless coding region between nucleotides −8 and +1298 (from −8 to +10, sense, 5′-GGAAGCCAATGGGCAGTG-3′ and from +1281 to +1298, antisense, 5′-GAATCAGCCAGTCATATC-3′) was subsequently used to isolate a 1306 b.p. fragment (see Methods).

Determination of the complete nucleotide and predicted amino acid sequences of the IRK1 insert revealed a one nucleotide change from the sequence reported by Kubo *et al.* (1993*a*; see Fig. 1*A*) which resulted in a conservative amino acid substitution of one non-polar residue for another: Met-203, encoded by ATG (Kubo *et al.* 1993*a*), was determined as Ile-203, encoded by ATA. In agreement with Kubo *et al.* (1993*a*), amino acid 289 was shown to be Asn, encoded by AAT (a codon for Asn) but not ATT

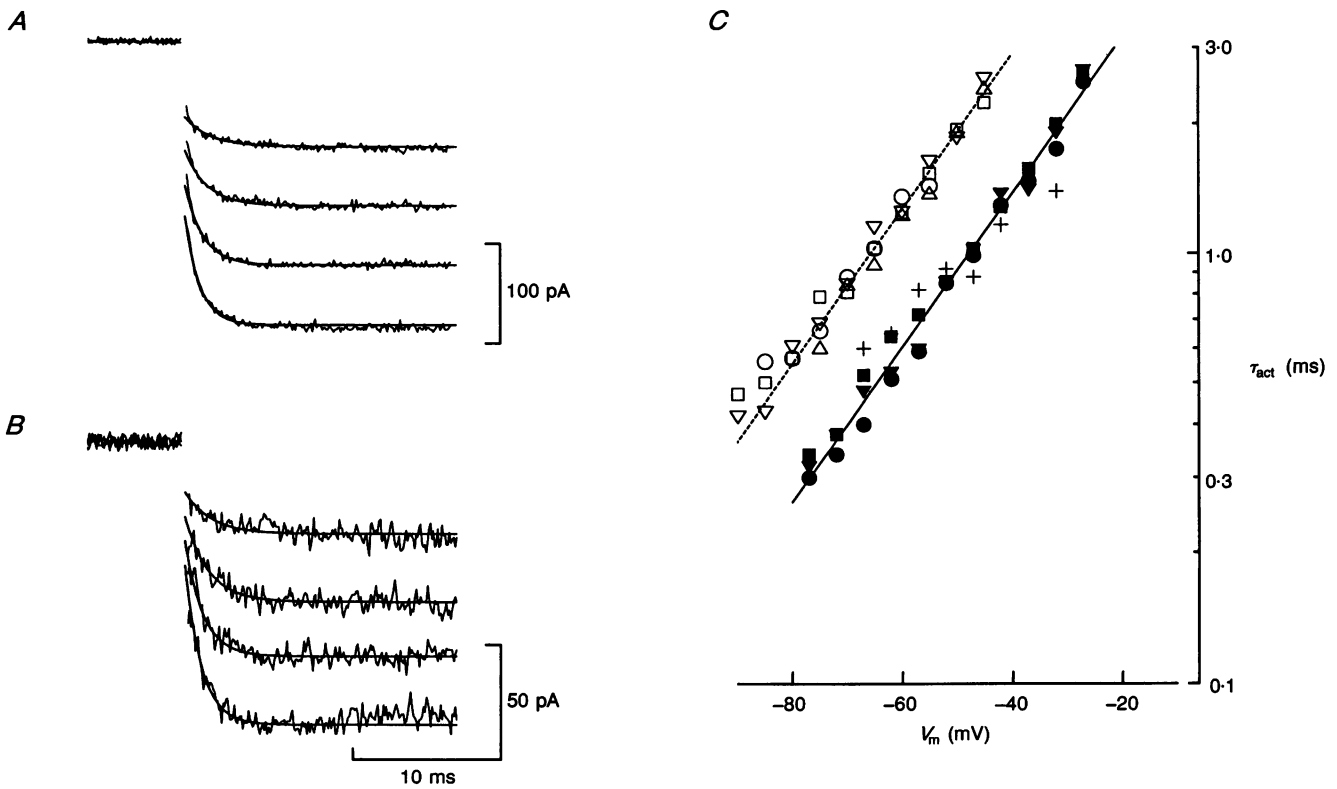

Figure 2. Activation of inward rectifier currents under hyperpolarization
A and *B*, records of currents under hyperpolarizations of 15, 20, 25 and 30 mV from E_K, fitted with single exponentials, for an experiment with 70 mM (*A*) and 35 mM $[K^+]_o$ (*B*). *C*, time constants for activation (τ_{act}; log scale, ordinate) plotted against voltage (V_m; abscissa) for experiments with 70 mM $[K^+]_o$, 1 mM $[Mg^{2+}]_i$ (■, ● and ▼); 35 mM $[K^+]_o$, 1 mM $[Mg^{2+}]_i$ (▽ and ○); 70 mM $[K^+]_o$, zero $[Mg^{2+}]_i$ (+); and 35 mM $[K^+]_o$, zero $[Mg^{2+}]_i$ (△ and □).

(a codon for Ile). The Ser-124 codon was determined as TCA in all our isolates, rather than TCG as given by Kubo *et al.* (1993*a*; Fig. 1*A*). As described by Kubo *et al.* (1993*b*), residue 53 was His, encoded by CAT, rather than Arg, encoded by CGT. Otherwise, the two sequences were identical.

IRK1 expresses inward rectifier K^+ channels in MEL cells

Figure 1*B* and *C* shows the result of whole cell recording from MEL cells in which the IRK1 gene has been expressed. As anticipated with IRK1, currents are small in response to depolarizing voltage pulses from E_K, but inward currents are large. Outward currents show no detectable time dependence in the presence of intracellular Mg^{2+} (see below), but inward currents increase with time during the first few milliseconds of the voltage pulse.

The relationship between chord conductance ($g_K = I_K/(V - E_K)$) and membrane potential may be fitted with a Boltzmann expression, with conductance initially increasing e-fold for an 11·9 mV hyperpolarization (Fig. 1*D*). In 70 mM $[K^+]_o$, the maximum g_K expressed was 10·3 ± 3·3 nS ($n = 8$; mean ± S.E.M.), corresponding to approximately 3 mS cm^{-2} in a spherical MEL cell of 10 μm diameter. As Kubo *et al.* (1993*a*) have also described, and as occurs in native channels (Hagiwara & Yoshii, 1979; Leech & Stanfield, 1981), this relation shifts 17·5 mV with 35 mM rather than 70 mM $[K^+]_o$, corresponding to the 17·5 mV change in E_K (Fig. 1*D*).

Intrinsic gating of IRK1

Figure 2 shows experiments to measure the rate of activation of current through IRK1 when the membrane was hyperpolarized from E_K in both the presence and absence of intracellular Mg^{2+}. Gating appears essentially first order (Fig. 2*A* and *B*). Activation time constants fall e-fold for a 24·1 mV hyperpolarization in both 70 and 35 mM $[K^+]_o$. This voltage dependence is in the range reported by others (18 mV: Leech & Stanfield, 1981; 28·8 mV: Ishihara *et al.* 1989). The relationship between activation time constants and voltage is shifted 17·5 mV with the change in E_K, so that, as occurs in native inward rectifiers (Hagiwara & Yoshii, 1979; Leech & Stanfield, 1981; Pennefather *et al.* 1992), the gating process depends on $(V - E_K)$ rather than simply on voltage.

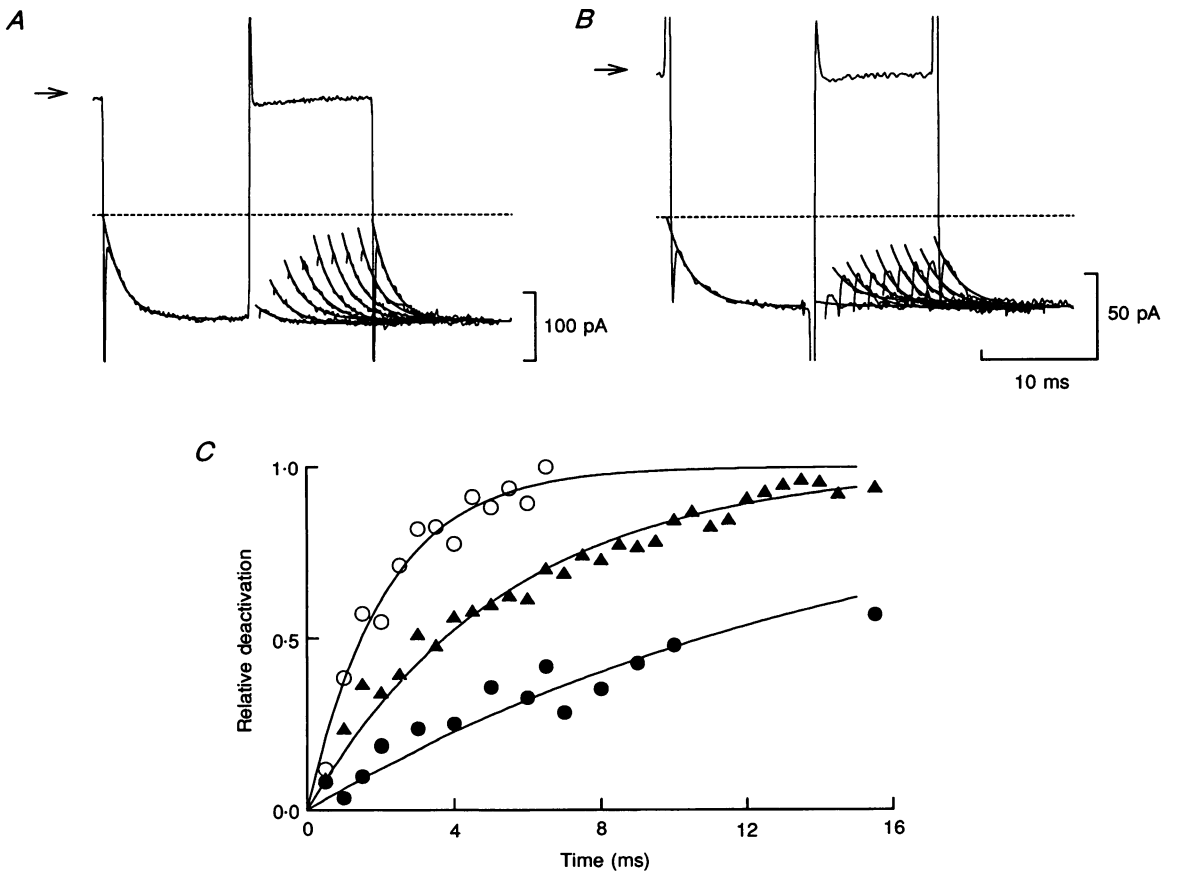

Figure 3. Reversal of activation at E_K; slowing by intracellular Mg^{2+}
A and *B*, records of currents obtained with $[Mg^{2+}]_i$ at zero (*A*) and at 1 mM (*B*). The arrows indicate the zero current level in each case; the horizontal dashed lines indicate the current at the start of the conditioning pulse. *C*, time course of deactivation measured with zero (○; $\tau_{deact} = 2{\cdot}1$ ms), 1 mM (▲; $\tau_{deact} = 5{\cdot}7$ ms) and 8·6 mM $[Mg^{2+}]_i$ (●; $\tau_{deact} = 15{\cdot}6$ ms), computed from initial currents found by fitting records as in *A* and *B* with an appropriate exponential function.

Effects of removal of intracellular Mg^{2+}

Removal of intracellular Mg^{2+} had little effect on the rate of activation of currents under hyperpolarization (Fig. 2), as Ishihara *et al.* (1989) have reported for native channels, though increasing $[Mg^{2+}]_i$ to 8·6 mM appeared to reduce the rate somewhat. However, removal of intracellular Mg^{2+} had two main effects on channel properties under depolarization. First, it speeded deactivation at these positive voltages (Fig. 3); and second it permitted outward currents to occur briefly after depolarization from E_K (Fig. 4). Such changes have been described previously in native channels (Matsuda *et al.* 1987; Vandenburg, 1987; Ishihara *et al.* 1989).

Figure 3 shows experiments in the presence and absence of intracellular Mg^{2+}, where the rate of the deactivation that occurs at E_K after a conditioning hyperpolarization was measured by applying test hyperpolarizations after varying intervals. Deactivation follows an exponential time course and is faster in the absence of intracellular Mg^{2+}, with the time constant for deactivation, $\tau_{deact} = 2·1$ and 2·4 ms in two experiments in zero $[Mg^{2+}]_i$ and 5·1 and 5·7 ms in 1 mM $[Mg^{2+}]_i$. This result has been interpreted as due to channels being unable to close while blocked by Mg^{2+} (Matsuda, 1988; Ishihara *et al.* 1989). Deactivation appears further slowed by raising $[Mg^{2+}]_i$ to 8·6 mM (Fig. 3*C*; $\tau_{deact} = 15·6$ and 11·6 ms in two experiments).

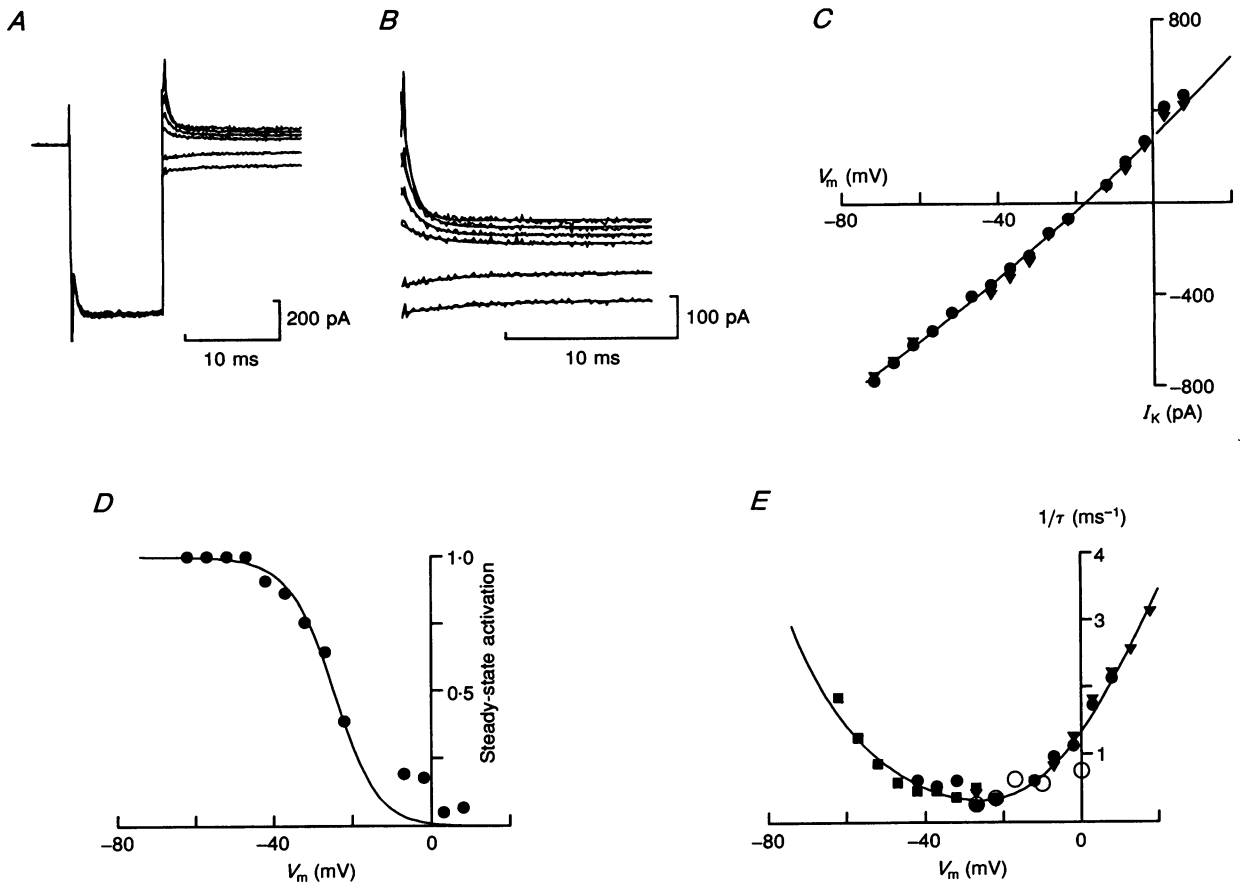

Figure 4. Gating of inward rectifier currents in the absence of intracellular Mg^{2+}
A and *B*, currents obtained on stepping by (from top) +20, +15, +10, +5, −5 and −10 mV from E_K after a 60 mV conditioning hyperpolarization. Currents are fitted with a single exponential function to find the rate of relaxation and the initial current; such fits are shown in *B*. *C*, instantaneous current–voltage relation fitted by a constant field expression (eqn (1) of text). *D*, modelling of steady-state activation of inward rectifier currents obtained from the current in the steady state after voltage steps as in *A* and *B* expressed as a fraction of the instantaneous current. A pedestal of 0·15, measured with large depolarizations, has been subtracted. *E*, rates of change of current under single hyperpolarizations (■), during measurements of recovery (○) and from experiments as in *A* and *B* (● and ▼). The lines in *D* and *E* give the fits from the model of Ishihara *et al.* (1989), with rate constants computed from:

$$\alpha = 0·17 \exp[-0·05(V_m - E_K)]/\{\exp[0·088(V_m - E_K)] + 1\} \text{ ms}^{-1},$$

$$\beta = 2·30 \exp[0·11(V_m - E_K - 15)]/\{\exp[0·085(V_m - E_K - 15)] + 1\} \text{ ms}^{-1},$$

with steady-state activation $= \alpha/(\alpha + \beta)$ (in *D*); rates of change of current $= \alpha + \beta$ (in *E*).

In order to investigate gating properties more fully in the absence of intracellular Mg^{2+}, a large outside-out patch was used (Fig. 4). Outward currents under depolarization now relax with time constants of approximately 1–2 ms or less. The outward transients are clearer after a preceding hyperpolarization from E_K, which fully activates the gating mechanism (Fig. 4*A* and *B*).

Figure 4*C* shows the instantaneous current–voltage relation obtained following such a prepulse by stepping back to various voltage levels, and by fitting the resulting current transients with exponentials to find the instantaneous current. In the absence of intracellular Mg^{2+}, the instantaneous current–voltage relation does not show inward rectification, but may be fitted well with the constant field expression:

$$I_K = P_K \frac{VF^2}{RT}\left(\frac{[K^+]_i \exp(VF/RT) - [K^+]_o}{\exp(VF/RT) - 1}\right), \quad (1)$$

with a P_K (permeability coefficient) for the patch of $4{\cdot}2 \times 10^{-11}$ cm^3 s^{-1}.

However, the gating still produces substantial inward rectification in the steady state, though we find a small pedestal of outward current under depolarization. Gating is illustrated in Fig. 4*D* and *E* and appears first order. Rate constants have been calculated from the increase in current under hyperpolarization and from the decline under depolarization; from current relaxations after a large hyperpolarization; and from experiments measuring deactivation following a hyperpolarizing conditioning pulse, as in Fig. 3. These methods produced identical values for the rate constant at a given voltage. We have chosen to describe our results by the model of Ishihara *et al.* (1989), after subtraction of the pedestal of outward current. Thus channels are considered to open and close as follows:

$$\text{Closed} \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} \text{Open}.$$

The rate constants (α and β) used to fit our experimental results are given in the legend to Fig. 4. The expressions used are the same as those given by Ishihara *et al.* (1989) with only slight modification of certain of the quantities (size of α and β; voltage-dependence of α; position on the voltage axis of β) in those expressions.

DISCUSSION

The results of this paper show that the IRK1 gene does not possess introns in its protein coding region and that it encodes a protein capable of forming inward rectifier potassium channels in a mammalian cell line, with the gating properties of native channels. Within experimental error, our results with cloned channels are similar to those obtained in cardiac myocytes by Ishihara *et al.* (1989). This gating process is present in spite of the absence of any obvious structure that could act as a voltage sensor (Aldrich, 1993); the membrane-spanning region M2 (Fig. 1*A*) contains a single negative charge (Asp at position 172), but this is unlikely to be sufficient solely to account for a gating process whose steady state suggests an effective valency substantially greater than 1. Given this strong voltage dependence of gating and the dependence of gating on the concentration of permeant K^+, we conclude that part of the effect of voltage is upon the binding of K^+ to some regulatory site of the kind initially proposed by Ciani, Krasne, Miyazaki & Hagiwara (1978) and developed further by Pennefather *et al.* (1992). Co-operativity of the kind also proposed by Ciani *et al.* (1978) and by Pennefather *et al.* (1992) would be necessary to account for the steep voltage dependence of the process.

REFERENCES

Aldrich, R. W. (1993). Potassium channels: advent of a new family. *Nature* **362**, 107–108.

Ciani, S., Krasne, S., Miyazaki, S. & Hagiwara, S. (1978). A model for inward rectification: electrochemical-potential-dependent gating of membrane channels. *Journal of Membrane Biology* **44**, 103–134.

Don, R. H., Cox, P. T., Wainwright, B. J., Baker, K. & Mattick, J. S. (1991). 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Research* **19**, 408.

Hagiwara, S. & Yoshii, M. (1979). Effects of internal potassium and sodium on the anomalous rectification of the starfish egg as examined by internal perfusion. *Journal of Physiology* **292**, 251–265.

Ho, K., Nichols, C. G., Lederer, W. J., Lytton, J., Vassilev, P. M., Kanazirska, M. V. & Hebert, S. C. (1993). Cloning and expression of an inwardly rectifying ATP-regulated potassium channel. *Nature* **362**, 31–38.

Ishihara, K., Mituiye, T., Noma, A. & Takano, M. (1989). The Mg^{2+} block and intrinsic gating underlying inward rectification of the K^+ current in guinea-pig cardiac myocytes. *Journal of Physiology* **419**, 297–320.

Kozak, M. (1984). Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. *Nucleic Acids Research* **12**, 857–862.

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

Kubo, Y., Reuveny, E., Slesinger, P. A., Jan, Y. N. & Jan, L. Y. (1993*b*). Primary structure and functional expression of a rat G-protein-coupled muscarinic potassium channel. *Nature* **364**, 802–806.

Leech, C. A. & Stanfield, P. R. (1981). Inward rectification in frog skeletal muscle and its dependence on membrane potential and external potassium. *Journal of Physiology* **319**, 295–309.

Matsuda, H. (1988). Open-state substructure of inwardly rectifying potassium channels revealed by magnesium block in guinea-pig heart cells. *Journal of Physiology* **397**, 237–258.

Matsuda, H., Saigusa, A. & Irisawa, H. (1987). Ohmic conductance through the inwardly rectifying K channel and blocking by internal Mg^{2+}. *Nature* **325**, 156–159.

Pennefather, P., Oliva, C. & Mulrine, N. (1992). Origin of the potassium and voltage dependence of the cardiac inwardly rectifying K-current (I_{K1}). *Biophysical Journal* **61**, 448–462.

Pongs, O. (1992). Molecular biology of voltage-dependent potassium channels. *Physiological Reviews* **72**, S69–88.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning – a Laboratory Manual*, 2nd edn. Cold Spring Harbor Press, New York.

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.

VANDENBURG, C. A. (1987). Inward rectification of a potassium channel in cardiac ventricular cells depends on internal magnesium ions. *Proceedings of the National Academy of Sciences of the USA* **84**, 2560–2564.

Acknowledgements

We thank Debra Langton for oligonucleotide synthesis and Julia Bartley for DNA sequencing. This work is supported by ICI, the MRC and the Royal Society. N.W.D. is a Royal Society University Research Fellow; P.A.S. held an SERC CASE research studentship during part of this work; I.A.K. is an SERC student.

Received 12 November 1993; accepted 1 December 1993.