Inwardly rectifying potassium channels expressed by gene transfection into the Green Monkey kidney cell line COS-1

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1. cDNA encoding a functional inwardly rectifying K^+ (IRK1) channel was transfected into COS-1 cells (a Green Monkey kidney cell line) using the liposome method, and voltage clamp experiments were done after 48–72 h.

2. Transfected cells showed inward rectification under whole-cell recording. The unitary current–voltage relationships in the inside-out configuration were almost linear in the absence of internal Mg^{2+} and polyamines, and the channel conductance averaged 34·1 ± 2·0 pS ($n = 15$) at 23–26 °C.

3. Internal Mg^{2+} (2–10 μM) induced sublevels in the outward current with one-third and two-thirds of the unitary amplitude as in native channels.

4. To determine the subunit stoichiometry, we constructed tandem multimeric cDNAs consisting of the coding sequences of the *IRK1* gene linked in a head-to-tail fashion. Cells transfected with tandem homomultimers up to octamers showed similar inwardly rectifying K^+ channels.

5. A mutation (E138Q) eliminated the ionic conductance of the channel. Channels expressed by dimeric constructs containing a single mutant have a conductance ranging between 5 and 35 pS.

6. The E138Q mutant cotransfected with a wild-type dimeric, trimeric or tetrameric construct did not alter the channel conductance. The results do not support the notion that IRK1 channel proteins consist of four subunits.

The phenomenon of inward rectification, whereby the K^+ conductance increases under hyperpolarization and decreases under depolarization, has been demonstrated in a variety of cell types (Katz, 1949; Hall, Hutter & Noble, 1963; Kandel & Tauc, 1966; Hagiwara & Takahashi, 1974). It is thought to play an important role in determining the resting potential and in permitting long depolarizing responses. Studies on native cardiac channels and cloned inwardly rectifying K^+ channels (Kubo, Baldwin, Jan & Jan, 1993) indicate that inward rectification is mainly the result of a voltage-dependent block of the channel pore by intracellular Mg^{2+} (Matsuda, Saigusa & Irisawa, 1987; Vandenberg, 1987; Matsuda, 1988) and polyamines (Ficker, Taglialatela, Wible, Henley & Brown, 1994; Lopatin, Makhina & Nichols, 1994).

The key feature of a Mg^{2+} block of the cardiac inwardly rectifying K^+ channel is that in the presence of internal Mg^{2+} at a micromolar level, the outward single-channel current fluctuates between four equally spaced conductance levels including zero current (Matsuda, 1988, 1991). Three distinct blocked states are also seen during blockage by internal Ca^{2+} (Matsuda & Cruz, 1993) and external Cs^+ or Rb^+ (Matsuda, Matsuura & Noma, 1989).

We confirmed that internal Mg^{2+} induces sublevels in channels expressed by transfecting COS-1 cells with IRK1 channels. To investigate the structural basis of substate behaviour, we studied the subunit stoichiometry of the inwardly rectifying K^+ channel using engineering constructs containing two to eight *IRK1* genes linked in a single open reading frame. In addition, we generated IRK1 channels with a mutation in the H5 region (E138Q), which abolished the ionic conductance. The present results did not support the notion that IRK1 channel proteins consist of four subunits.

METHODS

Mutagenesis and construction of tandem multimers

The *IRK1* gene inserted into pcDNAI/Amp (Invitrogen, San Diego, CA, USA) was digested with *Hind*III and *Stu*I to remove long 3′ untranslated sequences. The *Hind*III–*Stu*I fragment (~1·8 kb) which contains 5′ untranslated, coding and remaining 3′ untranslated sequences, was inserted into pTZ19R vector

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(Pharmacia, Uppsala, Sweden) for mutagenesis or the construction of tandem multimers. Site-directed mutagenesis of the cDNA was performed by means of a Transformer Site-Directed Mutagenesis Kit (Clontech, Palo Alto, CA, USA) using T4 DNA polymerase. The mutation was verified by restriction enzyme digestion and sequencing.

To construct dimeric cDNA, two *Pst*I sites were separately introduced by site-directed mutagenesis at the 5′ untranslated sequences adjoining the initiation codon and at the 3′ untranslated sequences including the third position of the termination codon. The two mutant cDNAs were ligated at the *Pst*I site. The amino acid sequence of the linking region between two subunits was Ile(428)-Trp-Leu-Gln-Pro-Met(1). To form trimeric cDNA, we constructed a cDNA having *Pst*I sites at both the 5′ and 3′ untranslated sequences and inserted it into the dimeric cDNA. To construct tetrameric–octameric cDNAs, vectors containing tandem multimeric cDNA were partially digested by *Pst*I, recovered by agarose gel electrophoresis, then ligated with the cDNA having *Pst*I sites at both termini. *Pst*I completely digested all the tandem multimer constructs, as expected. The direction of the inserted cDNAs in all the constructs was confirmed by restriction enzyme (*Bgl*II) digestion. When preparing plasmids containing tandem multimeric cDNA, competent cells (maximum efficiency stable 2; Gibco BRL, Gaithersburg, MD, USA) were used to avoid the release of inserts composed of repeated sequences.

Transfection of COS-1 cells

Expression plasmids were constructed by subcloning a *Bam*HI–*Bam*HI fragment containing the 5′ untranslated, coding and short (~190 bp) 3′ untranslated sequences as well as multiple cloning sites, from pTZ19R into the pSVL vector (Pharmacia). COS-1 cells were transiently transfected with the expression plasmid (1 μg per 35 mm dish) using Lipofectamine (GibcoBRL) according to the manufacturer's protocol. Currents were recorded from cells 48–72 h after transfection.

Electrophysiology

Whole-cell and single-channel currents were recorded using a fire-polished patch electrode (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) with an EPC-7 patch clamp amplifier (List Electronic, Darmstadt, Germany). Pipettes were made from capillaries of hard borosilicate glass (Pyrex) and for single-channel recording, were coated near their tips with silicone to reduce electrical capacitance. Single-channel current (*i*) records were obtained from inside-out patches in high-K^+ solution or from cell-attached patches in Tyrode or high-K^+ solution. The electrode resistance ranged between 6 and 14 MΩ when filled with a pipette solution containing 150 mM KCl, 1 mM $CaCl_2$ and 5 mM Hepes (pH 7·4). Whole-cell currents (*I*) were recorded in Tyrode solution using electrodes filled with high-K^+, Mg^{2+}-free solution (electrode resistance, 2–5 MΩ). Tyrode solution contained (mM): NaCl, 140; NaH_2PO_4, 0·33; KCl, 5·4; $CaCl_2$, 1·8; $MgCl_2$, 0·5; Hepes, 5; and glucose, 5·5; pH was adjusted to 7·4 with NaOH. High-K^+ solution contained (mM): potassium aspartate, 60; KCl, 65; KH_2PO_4, 1; $MgCl_2$, 0–3·62; EDTA, 2–5; K_2ATP, 3; and Hepes, 5; the pH was adjusted to 7·4 with KOH. The free Mg^{2+} concentration was calculated by means of a computer program (Fabiato & Fabiato, 1979; Tsien & Rink, 1980) using the dissociation constants (Martell & Smith, 1974) corrected for temperature and ionic strength (Harrison & Bers, 1989). Experiments were carried out at 23–26 °C.

Data were collected on digital audiotape using a PCM data recorder (TEAC, RD-101T, Tokyo, Japan) and stored for subsequent computer analysis (NEC, PC-98 XL, Tokyo, Japan). The unitary currents induced by the step pulses were filtered using a four-pole low-pass Bessel filter (NF, FV-665, Kanagawa, Japan) with a −3 dB corner frequency of 1·2 kHz and sampled at 5 kHz. The steady-state currents were filtered and digitized at 1 kHz. The membrane potentials were corrected for the liquid junction potential at the tip of the patch pipette in Tyrode solution and for that at the tip of the indifferent reference electrode filled with

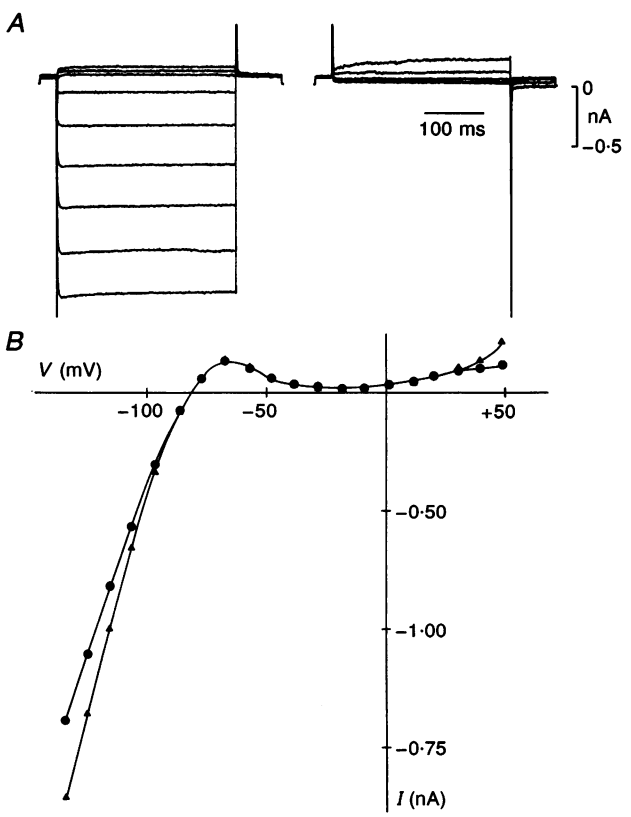

Figure 1. Whole-cell currents recorded from COS-1 cells transfected with *IRK1*

A, superposed current records were elicited by 300 ms hyperpolarizations from −57 to −136 mV in 10 mV increments (left-hand panel) and by depolarizations from −29 to +49 mV in 20 mV increments (right-hand panel). Holding potential, −48 mV. *B*, current–voltage (*I–V*) relationships obtained from the same cell. ●, current amplitude at the end of the capacitive current on the onset of test pulse; ▲, current amplitude at 280–290 ms of the test pulse.

Tyrode solution and placed in the bathing solution (Matsuda, 1991). The membrane capacitance determined from the ratio of the area under the capacitive current transient and the magnitude of the voltage step (10 mV hyperpolarization from a holding potential of −48 mV) was 7·2 ± 2·3 pF ($n = 20$).

Averaged results throughout this paper are given as means ± S.D. Student's unpaired t test was performed and P values of less than 0·05 were regarded as being statistically significant.

RESULTS

Mg^{2+} induced subconductance levels in cloned inwardly rectifying K^+ channels

Transfected cells showed inward rectification under whole-cell recording (Fig. 1). Outward currents were small and were not detectably time dependent. Large inward currents, increasing with time during the first several milliseconds, were induced by voltage steps to levels more negative than the equilibrium potential for K^+ (E_K). The current reversed at −82·3 ± 5·8 mV ($n = 10$). The region of negative slope conductance was observed at around −50 mV in the whole-cell current–voltage (I–V) relationship. The whole-cell conductance measured between −100 and −130 mV, where the I–V relationship was almost linear, was 6·4 ± 2·0 nS pF^{-1} ($n = 10$). Non-transfected cells showed outward rectification (Fig. 6A).

Figure 2 shows the single-channel currents recorded in the inside-out configuration in the absence of internal divalent cations and polyamines. E_K, predicted from a 150 mM external and internal K^+ concentration, was 0 mV. Inward currents show long-lasting openings characteristic of the inwardly rectifying K^+ channel. Outward currents were recorded in a steady-state condition at potentials more negative than +40 mV. At more positive potentials, outward currents were induced by stepping the membrane potential to positive values across E_K. The unitary i–V relationships were almost linear and the channel conductance averaged 34·1 ± 2·0 pS ($n = 15$) at 23–26 °C. This value was close to that of the cardiac inwardly rectifying K^+ channel obtained at the same K^+ concentration and temperature (Matsuda *et al.* 1989; Matsuda, 1991).

The phenomenon of inward rectification depends in part on a voltage-dependent block of channels by internal Mg^{2+} (Matsuda *et al.* 1987; Vandenberg, 1987). Subconductance levels with one- and two-thirds of the unitary amplitude of the outward currents were induced by low internal Mg^{2+} in the cardiac inwardly rectifying K^+ channel (Matsuda, 1988, 1991). Similar sublevels were also induced in channels expressed by transfection with *IRK1*. In the absence of internal Mg^{2+}, full-size single-channel currents were generated upon depolarization, usually followed by a closed

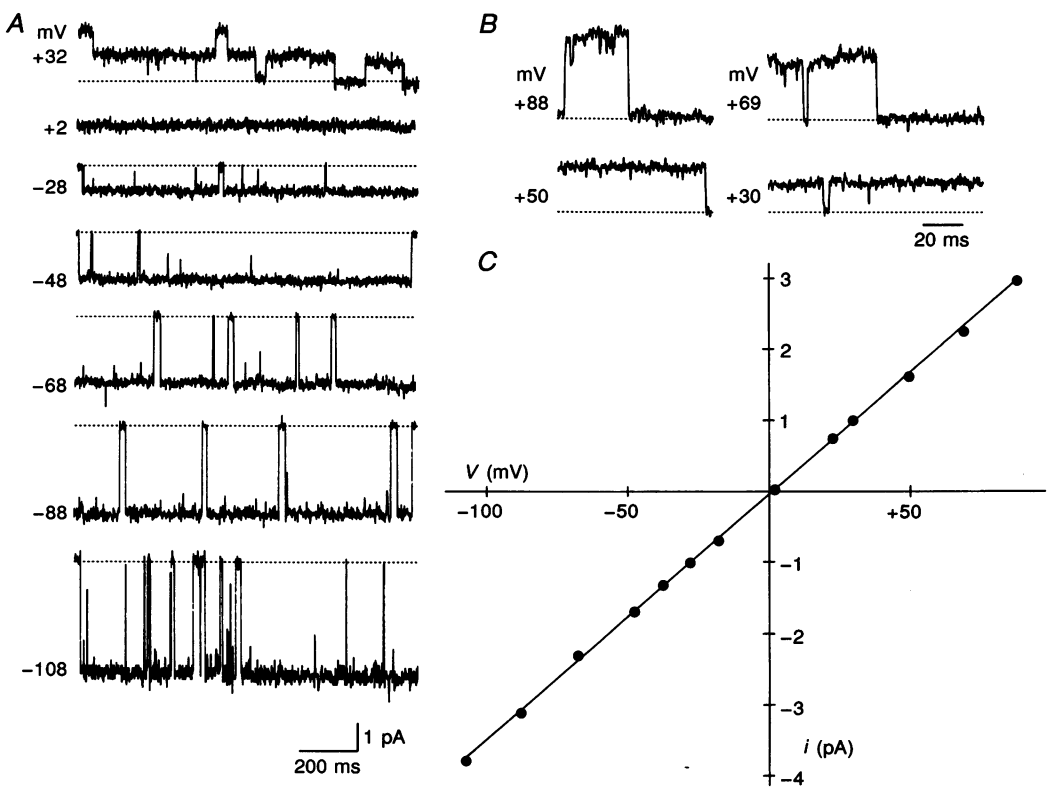

Figure 2. Single-channel currents recorded from IRK1 channels expressed in COS-1 cells in the inside-out configuration

Numbers to the left of each current trace refer to the holding potential (*A*), or the potential levels during the depolarizing steps from −48 mV (*B*). The dotted line indicates the zero-current level. Mg^{2+}-free internal solution. *C*, single-channel i–V relationship obtained from the same patch. The slope conductance of the unitary current was 34·6 pS.

state (or a blocked state by polyamines; Ficker *et al.* 1994) during a 130 ms pulse (Fig. 2*B*). While blocked by 2–10 μM Mg^{2+}, the outward open channel showed two, equally spaced discrete subconductance levels as indicated by the dashed lines, and it fluctuated between four levels including these sublevels (Fig. 3*A*). With increasing Mg^{2+} concentration or depolarization of the membrane, the channel stayed at the lower levels more frequently and the fluctuations became faster.

The open-state occupancies of each current level (the fraction of the total time for which the current stayed at each level) of cardiac inwardly rectifying K^+ channels are in reasonable agreement with the binomial theorem (Matsuda, 1988; Matsuda *et al.* 1989; Matsuda & Cruz, 1993). To determine whether or not the occupancies at current levels of zero (P_0), one-third (P_1), two-thirds (P_2) and fully open (P_3) conform to a binomial distribution in the block of IRK1 channels by internal Mg^{2+}, current records with the single-channel activity were reconstructed and the time spent at each of the four levels was measured (Matsuda, 1988). Zero-current periods longer than 20 ms, which were observed infrequently with internal Mg^{2+}, were attributed to the closed state of the channel (or the blocked state by internal polyamines remaining 0·5–1 h after isolation) and excluded from the analysis. The open probability of conducting units (p) was calculated on reconstructed traces by dividing the mean open-channel current by the unitary amplitude. Figure 3*B* shows the occupancies of each current level and the values predicted by the binomial theorem (arrowheads). (The equations representing occupancies are given in the figure legend.) Occupancies at each level essentially follow a binomial distribution.

Channels expressed by IRK1 tandem multimers

Having confirmed the three distinct blocked states by internal Mg^{2+} in cloned inwardly rectifying K^+ channels, we investigated the structural basis of this phenomenon. To determine the subunit stoichiometry of the IRK1 channel, we constructed tandem multimeric cDNAs consisting of the coding sequences of *IRK1* linked in a head-to-tail fashion.

cDNAs containing two to eight wild-type *IRK1* genes expressed channels indistinguishable from those expressed by the wild-type monomer. It has been reported that on a voltage-dependent K^+ channel, trimer expression gave a current that was around one-twentieth of that expressed by the tetramer for a similar concentration of cRNA (Liman, Tytgat & Hess, 1992). We measured the whole-cell conductance between −100 and −130 mV, where the *I–V* relationship was almost linear. The values obtained in cells transfected with wild-type trimeric (WT3) or tetrameric (WT4) cDNAs did not significantly differ ($12·8 \pm 6·9$ ($n = 11$) and $18·1 \pm 10·9$ nS pF^{-1} ($n = 11$), respectively).

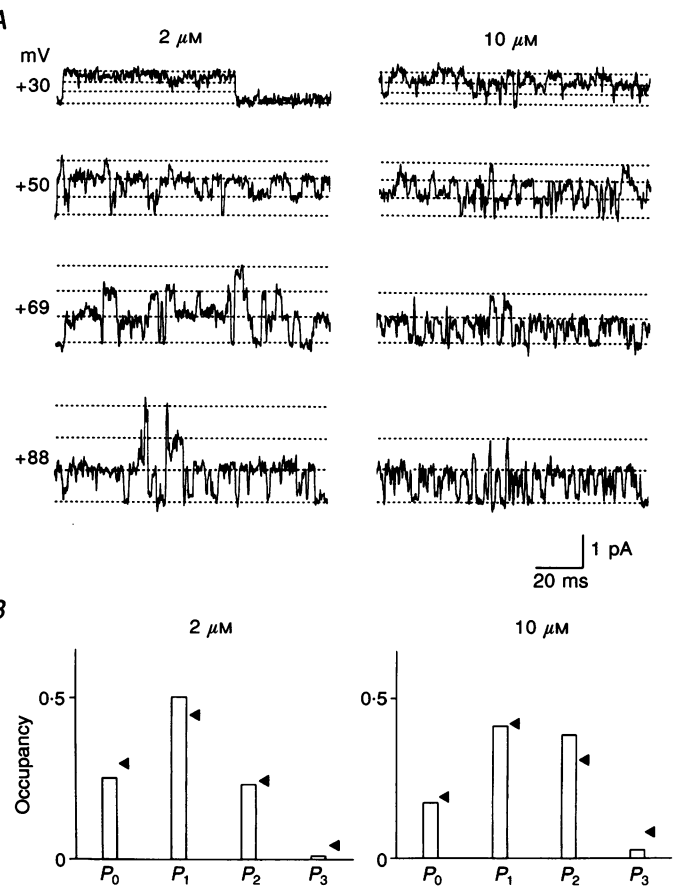

Figure 3. Sublevels induced in the outward current by internal Mg^{2+}

A, outward currents at different voltages in the presence of internal Mg^{2+}. The dashed lines indicate from the bottom, current levels for the zero, one-third, two-thirds and fully open channels. *B*, open-state occupancies of four current levels. A threshold level for the discrimination of open and blocked substates was set at around half of the sublevel. Arrowheads represent the values given from the following equations: $P_0 = (1 - p)^3$, $P_1 = 3p(1 - p)^2$, $P_2 = 3p^2(1 - p)$ and $P_3 = p^3$. Values for p and holding potential are 0·33 and +69 mV for 2 μM Mg^{2+} and 0·42 and +50 mV for 10 μM Mg^{2+}, respectively.

Figure 4 shows single-channel current records and unitary *i–V* relationships in the inside-out configuration in the channels expressed by transfection with WT3 and WT4. The channel conductance averaged 33·6 ± 1·6 pS ($n = 14$) for WT3 and 33·3 ± 1·9 pS ($n = 19$) for WT4, respectively. Similar sublevels were induced by internal Mg^{2+} to those in native channels and expressed by the wild-type monomer.

Since the dimeric construct (WT2), WT3 and WT4 expressed the IRK1 channel, we speculated that the number of subunits composing the IRK1 channel would be twelve, being the lowest common multiple of three and four, and that wild-type pentameric (WT5), heptameric (WT7) and octameric (WT8) cDNA constructs could not express the channel. On the contrary, all constructs containing five to eight *IRK1* genes (WT5, WT6, WT7 and WT8; Fig. 5*A*) led to the expression of the inwardly rectifying K^+ currents with the same macroscopic (Fig. 5*B*) and microscopic properties formed by the wild-type monomers, WT2, WT3 and WT4. The functional expression of more tandem multimers than pentamers, suggested that subunits that were part of a single polypeptide or separate polypeptides could assemble to form functional K^+ channels.

Effects of mutation in the H5 region on K^+ channel conductance

The inwardly rectifying K^+ channel and the voltage-dependent K^+ channel differ markedly in their overall amino acid sequence but share high homology in a short stretch called the H5 region (Kubo *et al.* 1993). This region is critical for ion selectivity and permeation in the voltage-dependent K^+ channel (Kirsch *et al.* 1992; Heginbotham, Lu, Abramson & MacKinnon, 1994). To understand the functional importance of negative charges in this region, we neutralized the glutamate at position 138 by replacing it with glutamine (E138Q). All ten cells transfected with the mutant monomer showed slowly activating outward currents with depolarizing pulses and small time-independent inward currents with hyperpolarizing pulses like native (non-transfected) COS-1 cells (Fig. 6*A*). Both the initial and steady-state current–voltage relationships showed outward rectification (Fig. 6*B*). None of forty patches on cells transfected with E138Q showed the inwardly rectifying K^+ channel. On the other hand, eleven of thirteen cells transfected with wild-type monomer showed inward rectification under whole-cell recording and unitary currents

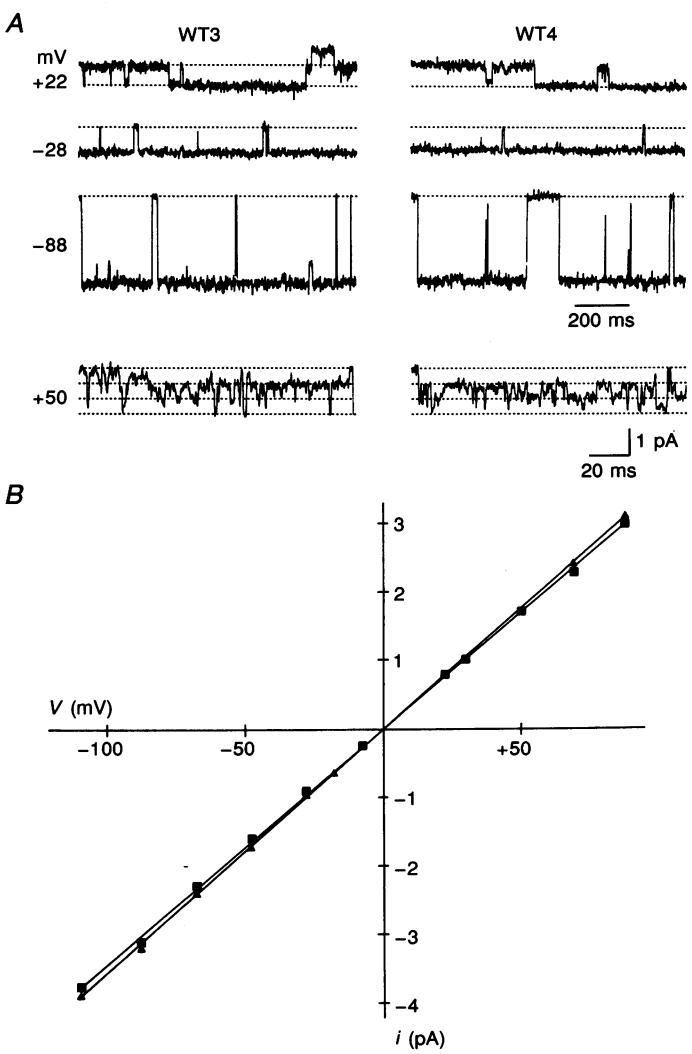

Figure 4. Channels expressed from wild-type trimeric (WT3) and tetrameric (WT4) cDNAs

A, single-channel currents recorded in the inside-out configuration in a steady-state condition without internal Mg^{2+} (upper three traces) and outward currents induced by voltage steps to +50 mV from −48 mV with 2 μM internal Mg^{2+} (bottom trace). *B*, unitary *i–V* relationships in the absence of internal Mg^{2+}. The slope conductance was 35·2 pS for the channels from WT3 (▲) and 34·3 pS for the channel from WT4 (■).

were recorded from fifty-four of ninety-two patches. It is thus suggested that the reduction of negative charges by the site-directed mutation abolishes current flow through the inwardly rectifying K^+ channel completely.

We considered that neutralization at position 138 of all subunits eliminated ionic conductance and that dimeric cDNAs containing one mutant together with one wild-type *IRK1* gene would express channels with a smaller conductance. Most channels expressed by dimeric constructs containing E138Q linked as the second unit to a wild-type *IRK1* gene (WT/E138Q) had smaller conductances, but some had the same conductance as those expressed by wild-type monomers or multimers (Fig. 7*A* and *B*). The unitary conductance of channels expressed by WT/E138Q ranged widely between 5 and 35 pS (Fig. 7*C*). This finding indicated that IRK1 channels were formed by subunits that are parts of separate polypeptides in the absence of a full complement of subunits on a single polypeptide (Liman *et al.* 1992; Yang, Jan & Jan, 1995*b*) and that mutant subunits reduced the channel conductance according to the number co-operating in the formation of the channel.

Cotransfection of E138Q with wild-type multimer

Although the above results suggested that functional channels could be formed by subunits that were parts of

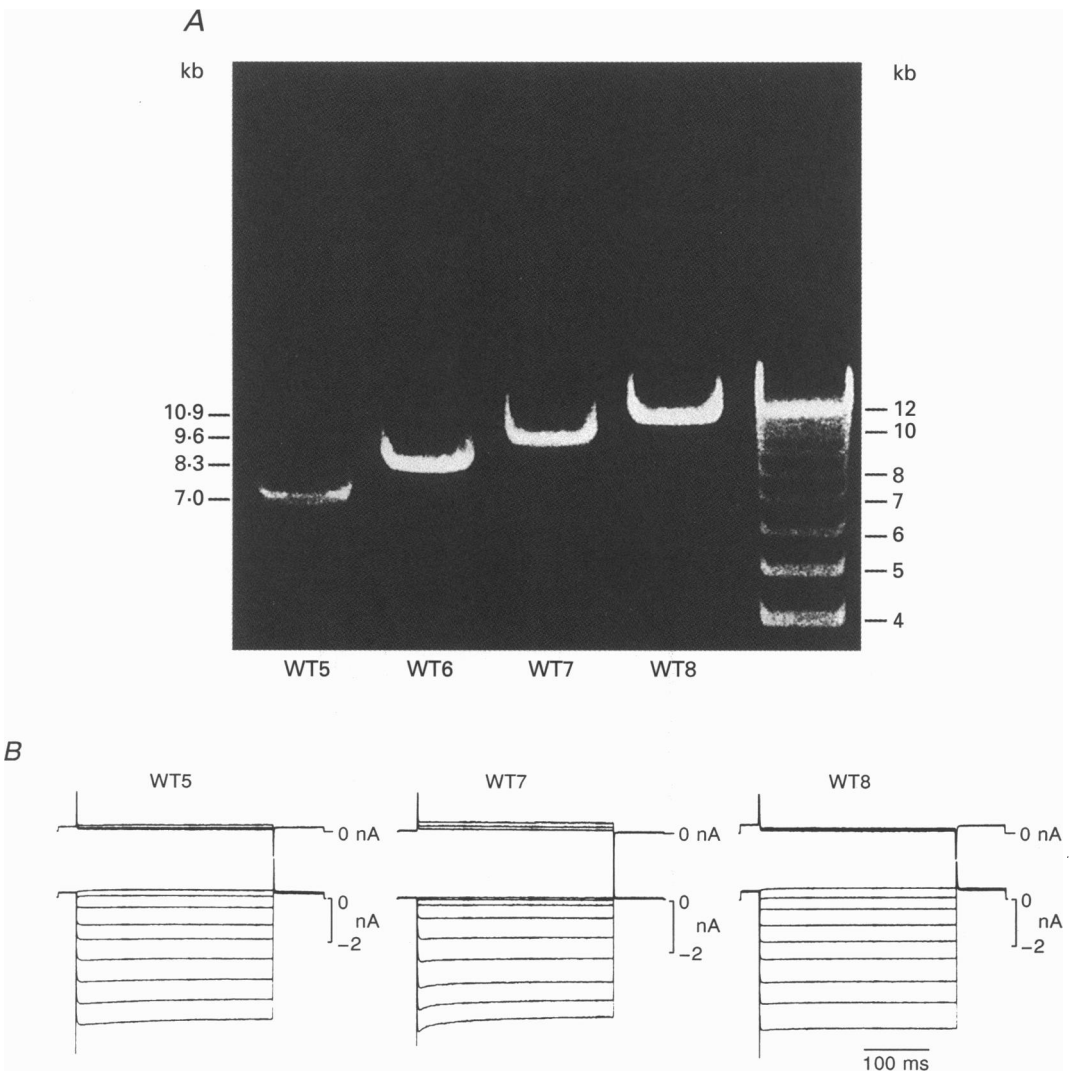

Figure 5. Channels were expressed from cDNA constructs containing five to eight wild-type *IRK1* genes (WT5–WT8)

A, *Eco*RI digestion of tandem multimeric cDNA. The pTZ19R vectors (~2·9 kb) each containing WT5, WT6, WT7 and WT8 were digested by *Eco*RI to release full-length tandem multimers. Digested fragments were separated on an agarose gel (0·7%). DNA bands over 4 kb are shown. The length of the DNA fragments increases by ~1·3 kb in proportion to the multiplicity. *B*, whole-cell currents recorded from cells transfected with WT5, WT7 and WT8. Superposed current records were elicited by 300 ms step pulses to −8, +20 and +50 mV (upper panel) and by hyperpolarizations from −67 to −147 mV in 10 mV increments (lower panel). Holding potential, −48 mV.

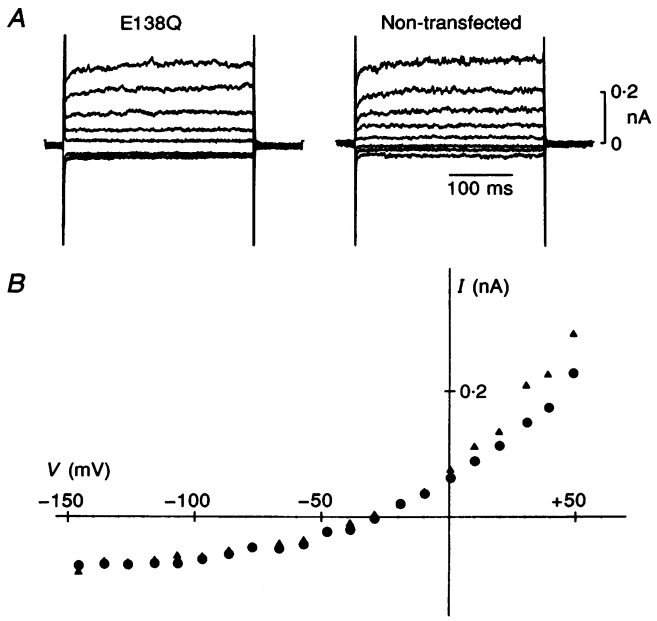

Figure 6. Mutation of E138Q abolished K^+ permeation

A, whole-cell currents recorded from a cell transfected with E138Q monomers were similar to those recorded from a non-transfected cell. Current traces correspond, from top to bottom, to clamp steps from a holding potential of −48 mV, to +49, +30, +10, −10, −29, −77, −107 and −136 mV. *B*, *I–V* relationships for a cell transfected with E138Q. ●, current amplitude at the end of the capacitive current on the onset of test pulse; ▲, current amplitude at 280–290 ms of the test pulse.

separate polypeptides, linking subunits into a single polypeptide chain will increase the relative concentration of the subunits to such a high level that most channels will be formed by the intramolecular assembly of the subunits on the same polypeptide chain. If so, the number of subunits composing the IRK1 channel protein could be estimated when the channel conductance was reduced by cotransfecting the E138Q monomer with a wild-type multimer, but not altered by cotransfection with a multimer containing one more *IRK1* gene. However, E138Q cotransfected with di-, tri- and tetrameric constructs did not alter the channel conductance (Fig. 8). Figure 8*A* and *B* shows current traces recorded in the cell-attached configuration and the unitary *i–V* relationships of cells in which E138Q was cotransfected

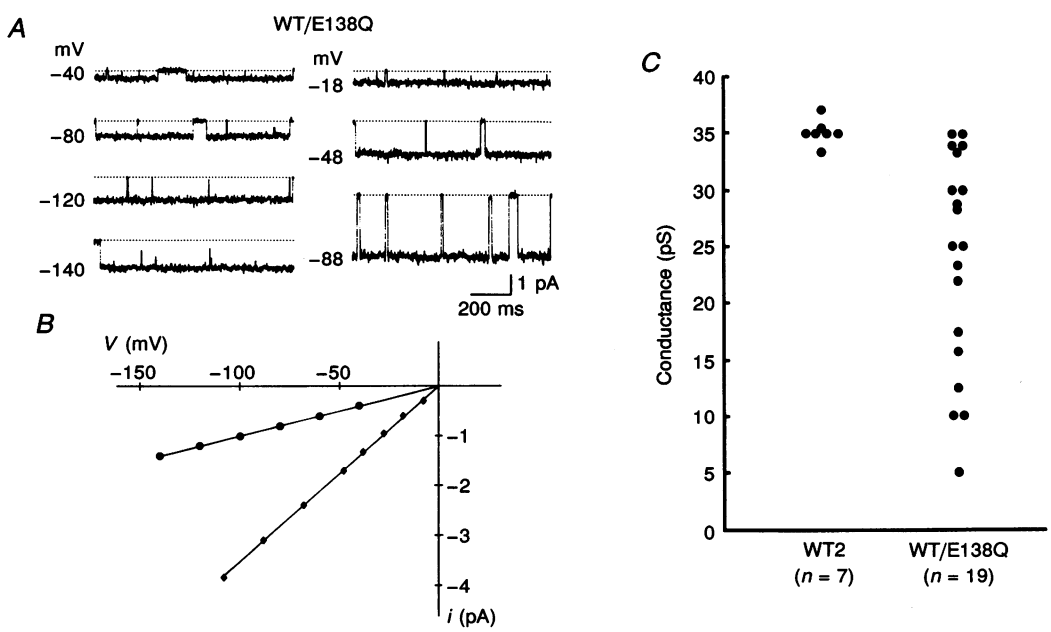

Figure 7. Channels expressed by dimeric constructs containing E138Q linked as the second unit to a wild-type *IRK1* gene (WT/E138Q)

A, left-hand panel shows steady-state currents recorded in the cell-attached configuration in Tyrode solution. The holding potential is expressed as the voltage deviation from the zero-current potential. Right-hand panel shows steady-state currents obtained from another cell in the inside-out configuration in high-K^+, Mg^{2+}-free solution. *B*, *i–V* relationships for the channels shown in the left panel (●) and right panel (◆) of *A*. The slope conductance was 10·0 and 35·2 pS, respectively. *C*, unitary conductance for channels expressed by wild-type dimeric constructs (WT2; 35·1 ± 1·1 pS; $n = 7$) and WT/E138Q.

with a trimer (WT3 + E138Q) and a tetramer (WT4 + E138Q). The conductance measured around the resting potential (V_{rest}) was 35·0 pS (WT3 + E138Q) and 34·1 pS (WT4 + E138Q), respectively. The results of the cotransfection experiments are shown in Fig. 8*C* together with the data for WT3 and WT4. The channel conductance did not differ under any circumstances.

DISCUSSION

This paper is the first description of single-channel currents through the IRK1 channel expressed in mammalian cells, COS-1 cells. The channel conductance of 34 ± 2 pS ($n = 15$) was substantially larger than that of channels expressed in *Xenopus* oocytes, 21 ± 2 pS ($n = 4$) at 22–25 °C with 140 mM external K^+ (Kubo *et al.* 1993). This value was closer to that of native cardiac inwardly rectifying K^+ channels (Matsuda *et al.* 1989; Matsuda, 1991). The reason for a difference in the single-channel conductance between COS-1 cells and oocytes remains unknown. There is a possibility that the IRK1 channel interacts with ATP-binding cassette (ABC) proteins like ATP-sensitive K^+ channels. ABC proteins and/or intermediate proteins such as cytoskeletal components (Higgins, 1995) may be different between oocytes and mammalian cells, altering the channel conductance.

This study showed that internal Mg^{2+} at micromolar levels induced sublevels with one- and two-thirds of the unitary amplitude in cloned channels as well as in native channels (Matsuda, 1988, 1991). Three distinct blocked states were also observed when the cardiac inwardly rectifying K^+ channel was blocked by internal Ca^{2+} (Matsuda & Cruz, 1993) and external Cs^+ or Rb^+ (Matsuda *et al.* 1989). The distribution of the current levels reasonably agreed with the binominal theorem at different probabilities for the blocked state (Matsuda, 1988; Matsuda *et al.* 1989; Matsuda & Cruz, 1993; and present study). The substate behaviour can be most simply explained by assuming that the inwardly rectifying K^+ channel consists of three identical conducting units that usually function co-operatively to form a single channel and that blocking ions enter and plug each conducting unit independently. Another mechanism that may induce sublevels is the binding of the blocking ions to a site (or sites) on the channel protein leading to different conductive states in a single-barrelled channel.

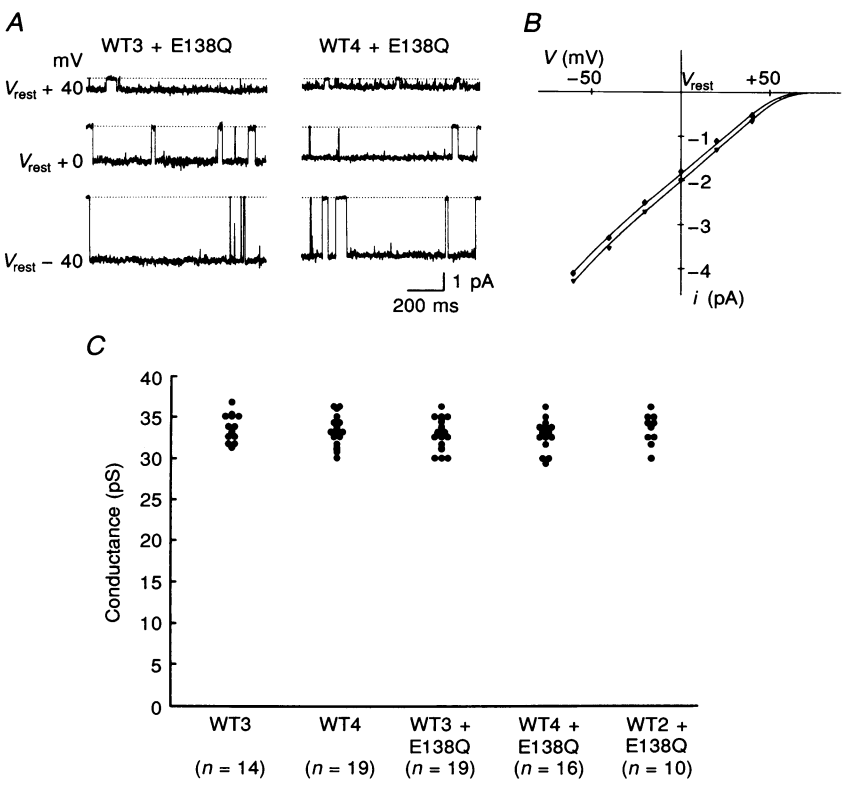

Figure 8. Cotransfection of E138Q and wild-type multimer

A, steady-state currents recorded from cells cotransfected with E138Q and WT3 (WT3 + E138Q) or WT4 (WT4 + E138Q). Cell-attached configuration. The holding potential is expressed as the voltage deviation from the resting potential (V_{rest}). *B*, *i–V* relationships for the channels expressed from WT3 + E138Q (▼) and WT4 + E138Q (◆). *C*, unitary conductance of channels expressed by WT3, WT4 and cotransfection of E138Q and WT2–WT4. The unitary conductance averaged 33·6 ± 1·6 pS (WT3), 33·3 ± 1·9 pS (WT4), 32·9 ± 1·9 pS (WT3 + E138Q), 32·8 ± 1·9 pS (WT4 + E138Q), and 33·5 ± 1·9 pS (WT2 + E138Q).

In the IRK1 channel, negatively charged aspartate at position 172 in the putative second transmembrane segment (Stanfield *et al.* 1994; Wible, Taglialatela, Ficker & Brown, 1994; Ficker *et al.* 1994; Yang, Jan & Jan, 1995*a*) and glutamate at position 224 in the putative cytoplasmic carboxyl domain (Taglialatela, Wible, Coporso & Brown, 1994; Yang *et al.* 1995*a*), contribute to the high affinity binding of Mg^{2+} and polyamines. Furthermore, a single wild-type subunit, along with three double-mutant subunits (D172N–E224G), retains high sensitivity to internal Mg^{2+} (Yang *et al.* 1995*b*). This is in contrast with the finding that reducing the negative charge in regions surrounding the segment M2 of one subunit affects the sensitivity to Mg^{2+} in the acetylcholine receptor channel (Imoto *et al.* 1988). Blocking the IRK1 channels with a mutation at D172 and/or E224 by internal Mg^{2+} has not been analysed at the single-channel level.

We investigated the functional importance of negative charges in the H5 region. Transfection with the mutant monomer (E138Q) did not induce the ionic conductance showing inward rectification, suggesting that neutralization at position 138 abolishes current flow through the IRK1 channel. Negatively charged glutamate at position 138 may form the site which K^+ ions binds to and thus be critical for K^+ ion permeation. On the other hand, tandem dimers containing one mutant and one wild-type *IRK1* gene expressed the IRK1 channel with a conductance ranging between 5 and 35 pS. The wide variance in the conductance is contrary to expectation that the conductance of IRK1 channels expressed by dimers with one mutant although constant, would be smaller than that expressed by wild-type monomers or multimers. The results suggested that IRK1 channels could be formed by subunits that were associated with separate polypeptides and that mutant subunits could reduce the channel conductance with the number co-operating in the formation of the channel.

Determination of the subunit stoichiometry of the IRK1 channel is considered to be the first step in understanding the molecular basis of the substates. We were unable to determine the number of subunits forming the channel by *IRK1* tandem multimers. Channels expressed by transfection with cDNAs containing two to eight wild-type *IRK1* genes were indistingushable from those expressed by the wild-type monomer at the whole-cell and single-channel level, indicating that the IRK1 channels could be formed by subunits that were part of a single polypeptide or separate polypeptides.

It has been reported that IRK1 channels (Yang *et al.* 1995*b*) and other strongly rectifying K^+ channels (BIR10; Glowatzki *et al.* 1995) are tetrameric, like voltage-gated K^+ channels (MacKinnon, 1991; Tytgat & Hess, 1992). Yang *et al.* (1995*b*) have examined the subunit stoichiometry by constructing tri- or tetramers with various numbers of double mutants and analysing the channels expressed in *Xenopus* oocytes. They found that twelve of thirteen channels recorded from the coexpressed WT4 and mutant monomer (D172N–E224K) were indistinguishable from the wild type, whereas eighteen of twenty-two channels from the coexpressed WT3 and mutant monomer were identical to fifteen of eighteen channels expressed by a tetramer containing one mutant, and concluded that IRK1 channels are tetrameric. In this study, cotransfection of wild-type multimers (WT2–WT4) with mutant monomer (E138Q) expressed channels similar to those expressed by wild-type monomer or multimers, arguing against the notion that the IRK1 channel consists of four subunits. Transcription *in vivo* (in COS-1 cells) and a low efficiency of expression of E138Q monomer may bring about results different from those of Yang *et al.* (1995*b*).

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