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Cloning and functional expression of the cDNA encoding a novel ATP-sensitive potassium channel subunit expressed in pancreatic β-cells, brain, heart and skeletal muscle

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Abstract A cDNA clone encoding an inwardly-rectifying potassium channel subunit (Kir6.2) was isolated from an insulinoma cDNA library. The mRNA is strongly expressed in brain, skeletal muscle, cardiac muscle and in insulinoma cells, weakly expressed in lung and kidney and not detectable in spleen, liver or testis. Heterologous expression of Kir6.2 in HEK293 cells was only observed when the cDNA was cotransfected with that of the sulphonylurea receptor (SUR). Whole-cell Kir6.2/SUR currents were K^+-selective, time-independent and showed weak inward rectification. They were blocked by external barium (5 mM), tolbutamide (K_d = 4.5 μM) or quinine (20 μM) and by 5 mM intracellular ATP. The single-channel conductance was 73 pS. Single-channel activity was voltage-independent and was blocked by 1 mM intracellular ATP or 0.5 mM tolbutamide. We conclude that the Kir6.2/SUR channel complex comprises the ATP-sensitive K-channel.

Key words: ATP-sensitive K-channel; Sulphonylurea receptor; Pancreatic β-cell; Kir6.2; Cardiac muscle; Skeletal muscle

1. Introduction

ATP-sensitive K-channels (K-ATP channels) are found in a variety of tissues including pancreatic β-cells, cardiac, smooth and skeletal muscle, axons and certain neurones [1,2]. Their most characteristic property is that they are inhibited by an increase in the intracellular ATP concentration: they thereby couple cellular metabolism to changes in transmembrane K-flux or electrical activity. K-ATP channels play important roles in both physiological (insulin secretion) and pathophysiological (cardiac and cerebral ischaemia) conditions [1–3]. Sulphonylureas, a class of drugs used in the treatment of non-insulin-dependent diabetes mellitus, act as potent inhibitors of K-ATP channels [4]. These drugs act by binding to a specific sulphonylurea receptor (SUR), which interacts with the K-ATP channel to bring about its closure. Cloning of a 140 kDa high-affinity sulphonylurea receptor (SUR) revealed it to be a member of the ATP-binding cassette (ABC) transporter family, with 2 nucleotide binding folds [5]. This protein does not show channel activity when expressed, suggesting SUR alone does not form the K-ATP channel.

The properties of the K-ATP channel suggest that it may belong to the superfamily of inwardly-rectifying potassium channels (Kir channels, [6]). At least six distinct subfamilies of Kir channels have been cloned. Like these cloned channels [6], the native K-ATP channel shows inward rectification, little time- or voltage-dependence, and is strongly K-selective [1,2]. None of the Kir channels cloned to date have a tissue distribution similar to that of the native K-ATP channel, suggesting that its molecular identity remains to be established. We have recently found that both Kir6.1 [7] and Kir1.1a [8] can couple to SUR (unpublished observations), suggesting they may share homology with the K-ATP channel. We therefore used these as probes to screen a β-cell cDNA library and isolated a novel Kir channel with a tissue distribution and biophysical properties corresponding to those of the native K-ATP channel.

2. Materials and methods

2.1. Molecular biology

We prepared a cDNA library from the mouse insulinoma cell line MIN6 using a ZAP Express cDNA synthesis kit (Stratagene): the average length of insert was 3 kb and the number of independent clones was 10^6. This library (5×10^5 phages) was screened at low stringency using mixed radiolabeled probes consisting of the full length cDNA of Kir6.1 and Kir 1.1a (hybridized at 37°C with 30% formamide and then washed at 0.3 × SSC, 0.1% SDS, 42°C for 1 h) and 4 positively hybridizing clones were isolated. Each isolated clone was excised using a helper phage (ExAssist) and recircularised to form a pBK-CMV vector containing the insert. Two independent clones were sequenced and found to be identical.

Total RNA was isolated from MIN6 and RINm5F (rat insulinoma) cells using TRI-REAGENT (Molecular Research Centre) and 10 μg of each total RNA, was separated on a 1% agarose-formaldehyde gel, transferred to a nylon membrane (Hybond-N, Amersham) and hybridised with Kir6.2 cDNA (entire coding region). We also probed a Multiple Tissue Northern Blot (Clonetech) containing 2 μg poly A^+ RNA per lane from various tissues with Kir6.2 cDNA. The blots were washed at high stringency (0.1 × SSC, 65°C for 1–2 h) and autoradiography was carried out for 2 days with intensifying screens.

2.2. Electrophysiology

HEK293 cells were transiently transfected with the pBK-CMV vector containing the coding sequence of Kir6.2, or hamster SUR ([5], subcloned into pcDNA3), using lipofectin (Life-Technologies), as described by the manufacturer. In most experiments we cotransfected HEK293 cells with Kir6.2 and SUR: the total DNA concentration was kept constant in cotransfections and the ratio of Kir6.2 to SUR was either 1:1 or 1:4. Mock-transfected cells received the same amount of plasmid without coding sequences, but were otherwise treated exactly the same. Cells were assumed to express Kir6.2 if their currents were larger than 1.96 standard deviations above the mean current (95% confidence limits) observed in mock-transfected cells in parallel transfections. For Kir6.2 + SUR transfected cells, this amounted to 23 out of 38 cells dialysed with 0.3 mM ATP and 5/8 cells dialysed with 5 mM ATP: only these cells were used for analysis.

Cells were cultured before and after transfection in MEM medium, supplemented with 10% FCS, 100 U/ml penicillin and 0.1 mg/ml streptomycin, at 37°C in an atmosphere of humidified air (95%) and CO_2 (5%). Whole-cell and single-channel currents were studied 48–72 h after

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transfection using the patch clamp technique. For whole-cell recordings, the pipette solution contained (mM): 107 KCl, 1.2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 5 HEPES (pH 7.2 with KOH; total K ~ 137 mM) and 0.3 mM ATP (or 5 mM ATP where indicated). The bath solution contained (mM): 40 KCl, 100 NaCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 5 HEPES (pH 7.4). Different K concentrations were obtained by equimolar substitution with NaCl. The holding potential was −30 mV. For inside-out patch recordings, the pipette contained (mM): 140 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 5 HEPES (pH 7.4) and the bath contained (mM): 107 KCl, 1.2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 5 HEPES (pH 7.2 with KOH; total K ~137 mM) plus MgATP and K_2ADP as indicated. Tolbutamide was dissolved in DMSO (final concentration <0.1%). Experiments were carried out at 22–25°C.

Drugs were tested once the whole-cell currents had reached a steady-state level (>15 min), with 0.3 mM $[ATP]_i$. Tolbutamide dose-response curves were obtained for each cell at all drug concentrations. Test solutions were alternated with control solutions and the current (I) is plotted as a fraction of the mean (I_c) of that obtained in control solution before and after exposure to the drug. Test solutions were applied in random order. Dose–response curves were fitted to a modified form of the Hill equation: $I/I_c = O + ((1 - O)/(1 + ([Tolb]/K_d)^n))$ where [Tolb] is the tolbutamide concentration, K_d is the tolbutamide concentration at which inhibition is half maximal, n is the Hill coefficient and O is an offset which describes the fact that in some cells the maximum block was not zero. Data are presented as mean ± S.E.M. and vertical lines indicate one SEM. Statistical significance was tested using the paired t-test.

Single-channel current recordings were filtered at 0.5 kHz or 2.5 kHz (for kinetics) by an 8-pole Bessel filter, and sampled at 1 or 10 kHz using a Digidata A/D converter (Axon Instruments). Channel activity was calculated as the mean current amplitude divided by the single-channel current for data stretches of 1–2 min. Mean open and closed times were obtained by fitting the lifetime distributions using a Simplex maximum likelihood method.

3. Results

A novel clone was isolated by screening a MIN6 cDNA library. The nucleotide sequence of this clone (Fig. 1A) predicts a protein of 390 amino acids (M_r = 43.561), sharing 74% homology with Kir6.1 (uK_{ATP} − 1 [7]), 48% with Kir1.1a (ROMK1 [8]), 50% with Kir2.1 (IRK1 [9]), 49% with Kir3.1 (GIRK1 [10]), 50% with Kir3.2 (BIR1 [11]) and 49% with Kir3.4 (rcK-ATP [12]). The homology to Kir6.1 suggests that this clone belongs to the same subfamily and should be named Kir6.2. In particular, like Kir6.1 [7], a GFG motif is found in the putative pore- forming loop instead of the GYG motif found in other Kir channels [7–12]. A hydrophobicity plot of Kir6.2 reveals two putative transmembrane domains, as found for other Kir channels. There are 2 potential cAMP-dependent phosphorylation sites (Thr-224 and Ser-372), 6 potential protein kinase C-dependent phosphorylation sites (Ser-3, Ser-37, Thr-190, Thr-336, Thr-345 and Ser-363) and 3 potential casein kinase II-dependent phosphorylation sites (Thr-62, Thr-224 and Ser-354). There is no obvious nucleotide consensus sequence for ATP-binding.

The tissue distribution of Kir6.2 mRNA is illustrated in Fig. 1B. Northern blot analysis showed two transcripts of 2.9 and 4.0 kilobases. These were strongly expressed in skeletal muscle, heart, brain and the insulinoma cells MIN6 and RINm5F. Weak expression was found in lung, very weak expression in kidney and no hybridization was detected in spleen, liver or testis.

HEK293 cells were transfected with Kir6.2, or cotransfected with Kir6.2 and SUR, cDNA and whole-cell currents examined using the standard configuration of the patch clamp method with 0.3 mM $[ATP]_i$. No difference was observed in the amplitude of whole-cell currents recorded in mock-transfected and Kir6.2 transfected cells, which were −249 ± 32 pA (n = 63) compared to −262 ± 66 pA (n = 25), respectively, at −100 mV. This suggests Kir6.2 does not express in HEK293 cells. In cells transfected with SUR alone the current amplitude was −291 ± 53 pA (n = 41) at −100 mV, which is not significantly different from mock-transfected cells and confirms that SUR does not itself form an ion channel. When cells were cotransfected with Kir6.2 and SUR, however, whole-cell currents were initially larger than in mock-transfected cells (−743 ± 165 pA) and further increased to −2621 ± 460 pA (n = 12, P < 0.001), at −100 mV, within the next 15 min (Fig. 2A). A similar effect is found in pancreatic β-cells where it has been attributed to the washout of ATP from the cell following dialysis with the pipette solution [13]. No such increase in current was observed over a 15 min period in mock-transfected cells, Kir6.2-transfected cells or SUR-transfected cells; or when cells cotransfected with Kir6.2 + SUR were dialysed with 5 mM ATP, current amplitudes at −100 mV being −659 ± 56 pA at 0 min and −564 ± 105 pA at 10 min (n = 5). This indicates that Kir6.2/SUR currents are inhibited by intracellular ATP.

Fig. 2B,C shows whole-cell currents and the associated current–voltage (I–V) relationship from a cell cotransfected with Kir6.2 and SUR. Currents activated instantaneously upon hyperpolarization, were time-independent and showed weak inward rectification, properties which are characteristic of native K-ATP channels [1,2]. The Kir6.2/SUR currents reversed at −23.7 ± 1.4 mV (n = 6) in 40 mM external K^+. Reduction of $[K^+]_0$ decreased the slope conductance and shifted the reversal potential to more negative potentials (Fig. 2D). The shift in the reversal potential was 45.2 ± 3.2 mV (n = 6) for a 10-fold change in $[K^+]_0$ (Fig. 2E), indicating that the channel is highly K-selective.

The pharmacology of Kir6.2/SUR currents was consistent with that of native K-ATP channels. Thus, 5 mM barium blocked the current in a time- and voltage-dependent manner, the current being blocked by 74.9 ± 10.6% (n = 7, P < 0.01) at −100 mV (Fig. 3A,D). Quinine (20 μM) inhibited Kir6.2/SUR currents by 68.9 ± 10.9% (n = 5, P < 0.02) at −100 mV (Fig. 3D), a potency consistent with that of native K-ATP channels [14]. Tolbutamide (0.5 mM) also blocked Kir6.2/SUR currents (Fig. 3B), by 68.7 ± 7.9% at −100 mV (n = 14, P < 0.01; Fig. 3D). A representative dose–response curve for tolbutamide inhibition is shown in Fig 3C and was fitted best with a K_d = 2.4 μM and a Hill coefficient of 1.04: mean values were 4.2 ± 1.4 μM and 0.91 ± 0.21 μM (n = 5), respectively. These values are similar to those found for K-ATP currents in pancreatic β-cells [1,2,4,13].

Fig. 4A shows single-channel currents recorded from an inside-out patch on a HEK293 cell cotransfected with Kir6.2 and SUR. At negative potentials, channel openings were clustered into bursts separated by long closed periods, whereas openings of longer duration were found for outward currents. During the burst, the mean open and closed times were 1.51 ± 0.27 ms and 0.38 ± 0.05 ms (n = 6) at −70 mV, respectively. There was no clear dependence of the open probability on membrane potential. The current–voltage relationship (Fig. 4B) showed weak inward rectification, the currents at +30 mV being ~53 ± 7% (n = 5) of those at −30 mV, and the mean single-channel conductance, measured over the linear part of the I–V relationship (from −10 to −70 mV), was 72.5 ± 2.2 pS (n = 8). In inside-out

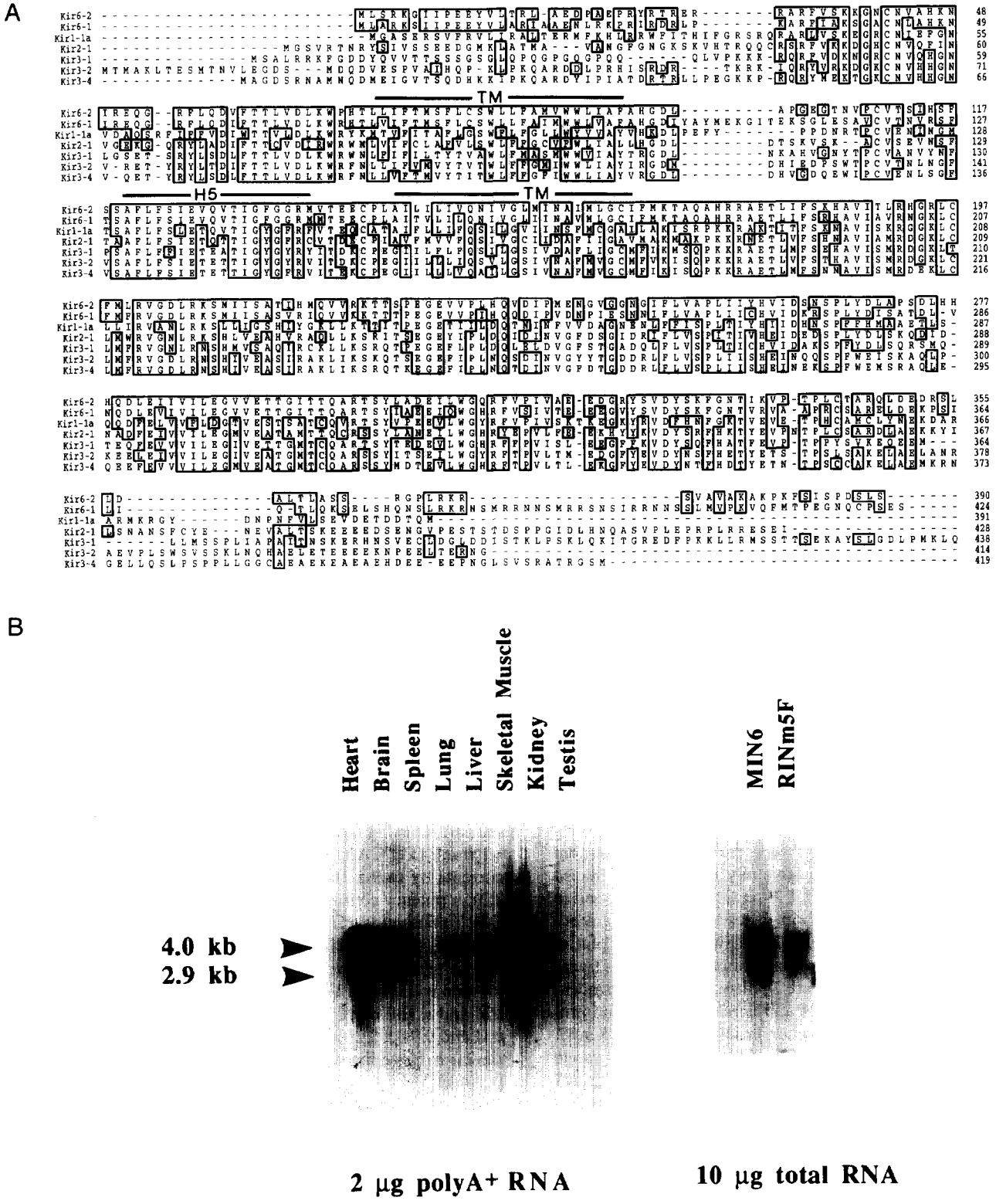

Fig. 1. (A) Predicted amino acid sequence of Kir6.2 and comparison with other members of the inward rectifier family. Residues shared with at least Kir6.2 are boxed; putative transmembrane (TM) and pore (H5) domains are overlined. Dashes represent minimal gaps introduced to maximize the comparison. (B) Tissue distribution of Kir6.2. Left: Poly A$^+$ RNA (2 μg/lane) from the specified tissues was hybridised with Kir6.2. Right: Total RNA (10 μg/lane) was extracted from MIN6 cells or RINm5F cells and hybridised with Kir6.2.

patches, single-channel currents were inhibited by application of 1–5 mM ATP (Fig. 4C and 3 others) or 0.5 mM tolbutamide (Fig. 4D). All these properties are characteristic of native K-ATP channels.

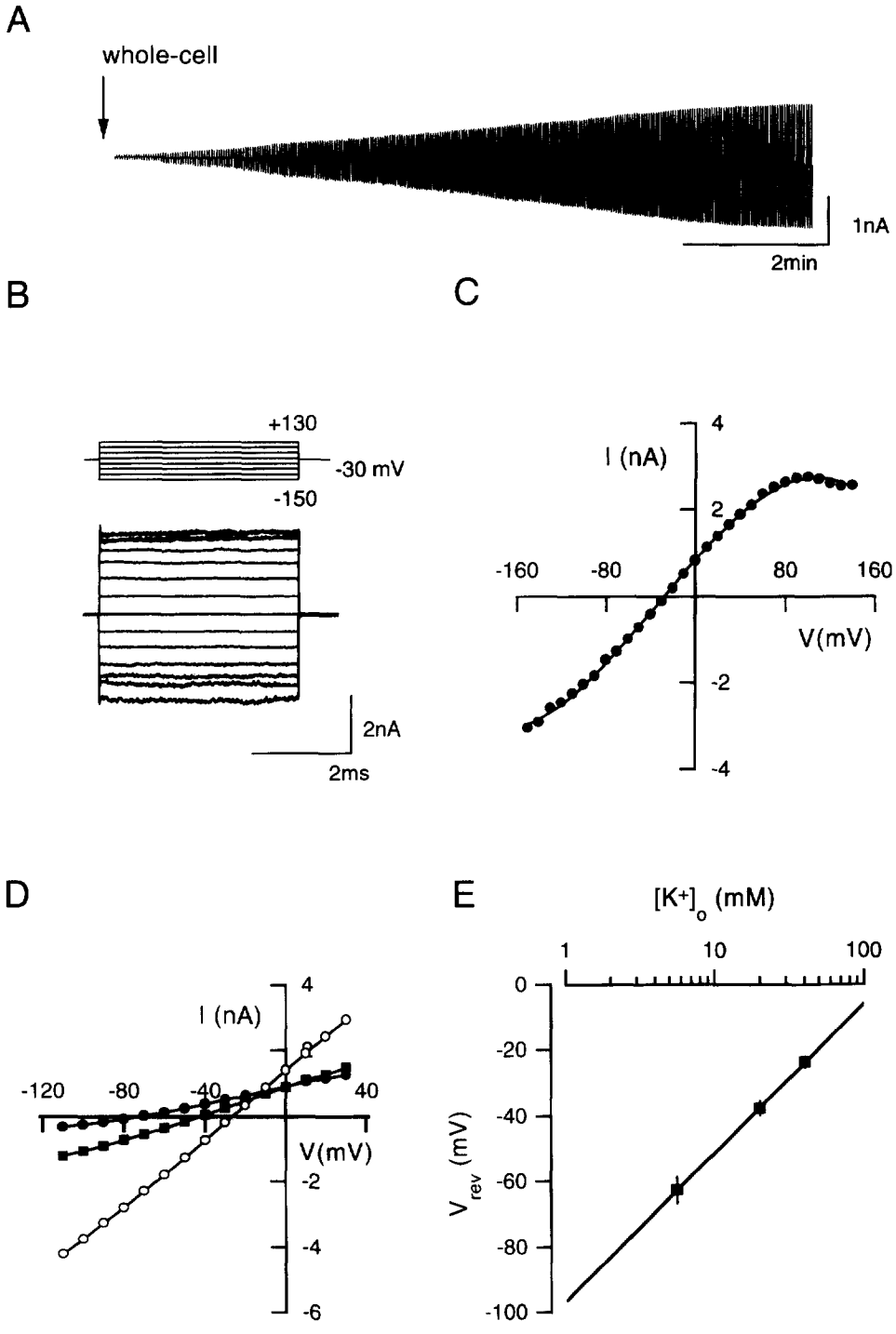

Fig. 2. (A) Whole-cell currents recorded from a HEK293 cell cotransfected with cDNA encoding Kir6.2 and SUR in response to alternate 20 mV depolarizing or hyperpolarizing pulses from a holding potential of −30 mV. (B,C) Whole-cell currents (B) and corresponding current–voltage (I–V) relationship (C) recorded from a HEK293 cell cotransfected with Kir6.2 and SUR, in response to a series of voltage steps from −150 to +130 mV from a holding potential of −30 mV. (D) Current–voltage (I–V) relationships recorded for the same cell in 40 mM K (○), 20 mM K (■) and 5.6 mM K (●) solution. Holding potential, −30 mV. (E) Mean relationship between the current reversal potential and $[K]_o$, for 6 cells. The line has a slope of 45 mV per decade change in $[K]_o$.

Single-channel currents were observed in 19/35 inside-out patches and at higher frequency in cell-attached patches (>80% of patches in some transfections). In most cases more than one channel was found in each patch. A channel with similar kinetics and conductance (67.8 ± 0.8 pS, n = 6) was observed occasionally in patches on mock-transfected cells (15/94 cell-attached patches; one channel per patch) but ran down rapidly after excision and was not inhibited by 1 mM ATP (6/6 patches).

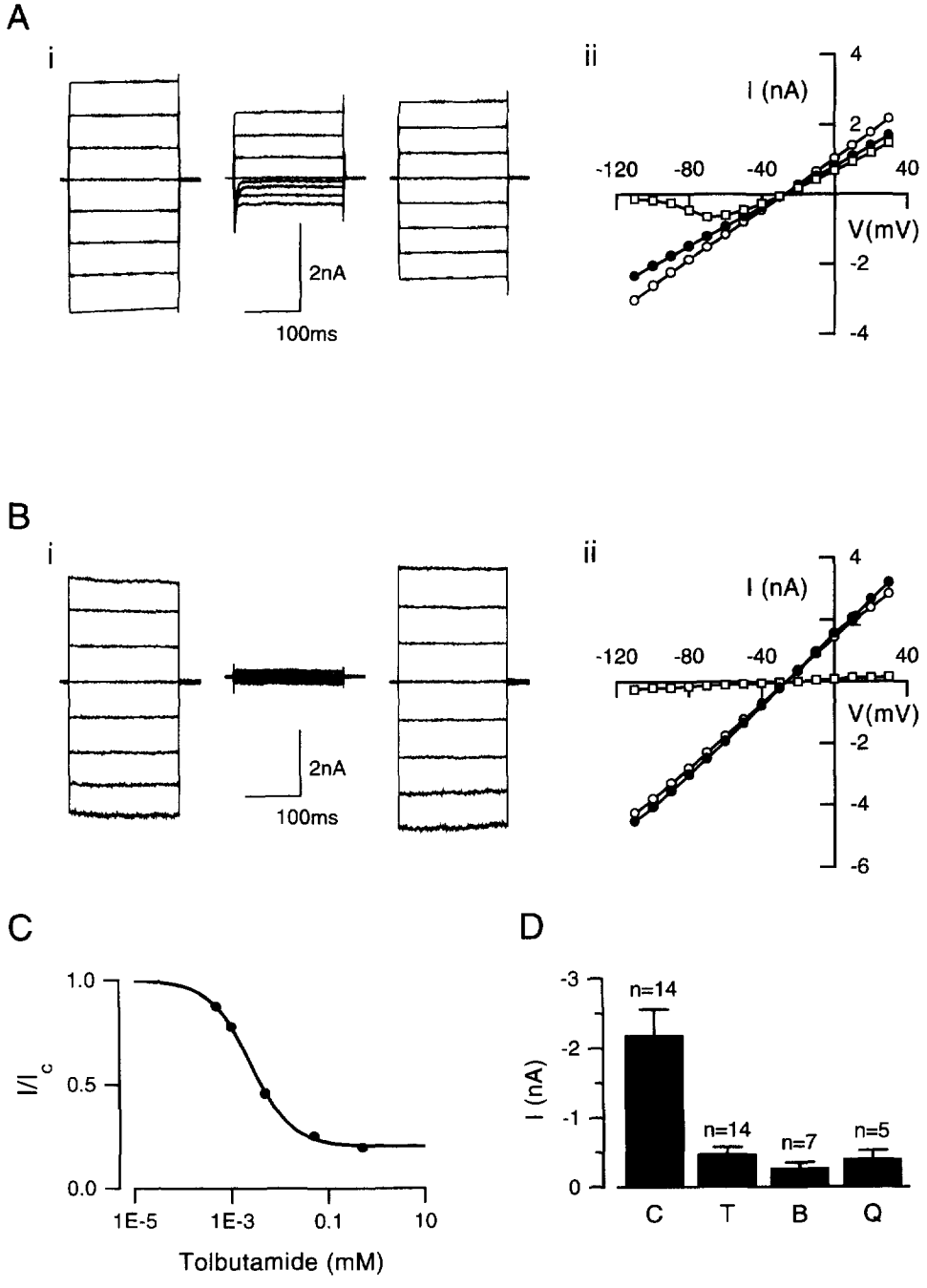

Fig. 3. (A,i) Whole-cell currents recorded before (left), during (centre) and after (right) addition of 5 mM barium to the external solution, from a HEK293 cell transfected with Kir 6.2 + SUR. (A,ii) *I–V* relationships recorded for the same cell in control solution (○, ●), or in the presence of 5 mM barium (□). (B,i) Whole-cell currents recorded before (left), during (centre) and after (right) addition of 0.5 mM tolbutamide to the external solution, from a HEK293 cell transfected with Kir 6.2 + SUR. (B,ii) Current–voltage (*I–V*) relationships recorded for the same cell in control solution (○, ●), or in the presence of 500 μM tolbutamide (□). (C) Relationship between tolbutamide concentration and the whole-cell current, expressed as a fraction of its amplitude in the absence of the drug, for a cell cotransfected with Kir6.2 + SUR. The line is the best fit of a modified Hill equation to the data, using least squares analysis, and gives K_d = 2.4 μM, n = 1.04 and O = 0.2. (D) Mean whole-cell Kir6.2/SUR currents recorded at −100 mV in control solution (C) and in the presence of 0.5 mM tolbutamide (T), 5 mM barium (B), or 20 μM quinine (Q). The number of cells is indicated above the bars.

4. Discussion

The results presented here demonstrate that Kir6.2 encodes an inwardly-rectifying ATP-sensitive K-channel subunit which is strongly expressed in pancreatic β-cells, brain, heart and skeletal muscle. This tissue distribution echoes that of the native K-ATP channel [1–2] and suggests that Kir6.2 may form part of this channel. A similar tissue distribution is found for sulphonylurea binding [4] implying that SUR and Kir6.2 may couple in native cells.

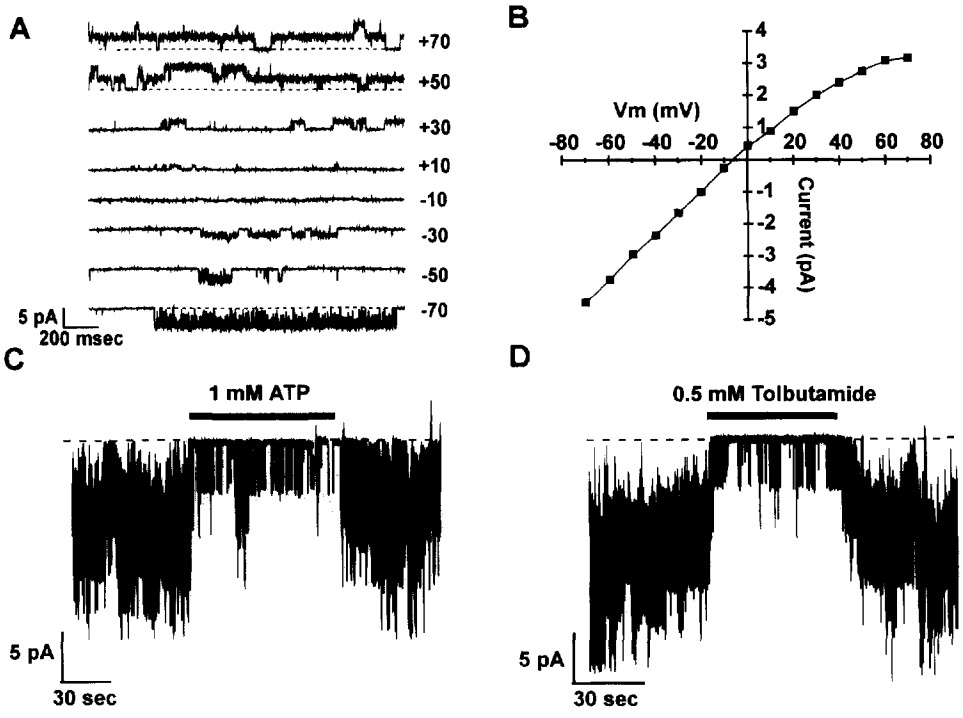

Fig. 4. (A,B) Single-channel currents (A) and corresponding current–voltage (I–V) relationship (B) recorded from an inside-out patch on an HEK293 cell cotransfected with Kir6.2 plus SUR. A. The dashed lines indicate the zero current level. The holding potential is indicated to the right of each trace. Filter frequency, 0.5 kHz; sample frequency, 1 kHz. B. The single-channel conductance was 71 pS (measured between −10 mV and −70 mV). (C,D) Single-channel currents recorded at −70 mV from the same inside-out patch as in A. 1 mM ATP (C) or 0.5 mM tolbutamide (D) were applied as indicated by the bars. Channel activity was reduced by 90% (C) and 97% (D). The dashed lines indicate the zero current level.

Although we were unable to detect a significant increase in whole-cell currents in HEK293 cells transfected with Kir6.2 alone, very large currents were observed when Kir6.2 was cotransfected with SUR. This suggests that Kir6.2 does not express functional channels in HEK293 cells and that it couples in some way to SUR to form a functional channel complex. It remains unclear whether SUR simply facilitates the insertion of Kir6.2 into the membrane, whether it is needed for channel activity or whether both processes are involved. Mutations in SUR result in persistent hyperinsulinaemia of infancy (PHHI), a disease associated with unregulated insulin secretion [15]. Our finding that SUR is essential for functional Kir6.2 activity suggests that mutations in SUR produce a loss of K-ATP channel activity in PHHI patients, which would be expected to depolarise the β-cell and thus cause insulin secretion in the absence of secretagogues. Indeed, β-cells from PHHI patients have been reported to lack K-ATP channel activity [16]. An obvious possibility is that mutations in either SUR or Kir6.2 may also account for the *decreased* insulin secretion observed in non-insulin-dependent diabetes mellitus.

Kir6.2/SUR currents show no time-dependent activation kinetics and weak inward rectification in the presence of 1.2 mM intracellular Mg^{2+}, consistent with the fact that the second transmembrane region contains an asparagine at position 160, and that position 212 is occupied by serine. The equivalent residues are negatively charged in strong inward rectifiers [17].

We observed a marked time-dependent increase in the amplitude of Kir6.2/SUR currents following dialysis with an intracellular solution containing 0.3 mM ATP, but not with 5 mM ATP, suggesting that the current is activated by the washout of ATP from the cell. We also observed direct inhibition of single-channel currents by ATP applied at the inner face of the membrane. Since Kir6.2 possesses no obvious consensus sequence for ATP-binding, and SUR has two ATP-binding domains [5], it is possible that ATP-sensitivity is conferred by the SUR subunit of the channel complex. However, in the absence of independent expression of Kir6.2 it will be necessary to mutate the nucleotide binding domains of SUR to verify this idea. Alternatively, a third protein, endogenously expressed in HEK293 cells, may confer ATP-sensitivity.

We have found that SUR can confer sulphonylurea sensitivity on other types of Kir channels, such as Kir1.1a and Kir6.1, although it is not essential for expression of these channels (unpublished observations). It therefore seems likely that the sulphonylurea sensitivity of Kir6.2/SUR currents is conferred by the SUR subunit of the complex.

The tissue distribution, single-channel conductance, kinetics, rectification properties, K-selectivity, inhibition by ATP and pharmacological properties of the Kir6.2/SUR complex are all consistent with those of native K-ATP channels. We conclude that the K-ATP channel consists of a complex of Kir6.2 and SUR, with Kir6.2 acting as the pore-forming subunit and SUR acting as a regulator subunit which confers sulphonylurea, and possibly also ATP-sensitivity.

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References

[1] Ashcroft, F.M. (1988) Annu. Rev. Neurosci. 11, 97–118
[2] Ashcroft, F.M. and Ashcroft, S.J.H. (1990) Cell Sig. 2, 197–214
[3] Heurteaux, C., Bertaina, V., Widmann, C. and Lazdunski, M. (1993) Proc. Natl. Acad. Sci. USA 90, 9431–9435.
[4] Ashcroft, F.M. and Ashcroft, S.J.H. (1992) Biochim. Biophys. Acta 1175, 45–59.
[5] Aguilar-Bryan, L., Nichols, C.G., Wechsler, S.W., Clement, J.P., Boyd, A.E., González, G., Herrera-Sosa, H., Nguy, K., Bryan, J. and Nelson, D.A. (1995) Science 268, 423–425.
[6] Doupnik, C.A., Davidson, N. and Lester H.A. (1995) Curr. Op. Cell Biol. 5, 268–278.
[7] Inagaki, N., Tsuura, Y., Namba, N., Masuda, K., Gonoi, T., Horie, M., Seino, Y., Mizuta, M. and Seino, S. (1995) J. Biol. Chem. 270, 5691–5694.
[8] Ho, K., Nichols, C.G., Lederer, W.J., Lytton, J., Vassilev, P.M., Kanazirska, M.V. and Hebert, S.C. (1993) Nature 362, 31–38.
[9] Kubo, Y., Baldwin, T.J., Jan, Y.N. and Jan, L.Y. (1993). Nature 362, 127–133.
[10] Kubo, Y., Reuveny, E., Slesinger, P.A., Jan, Y.N. and Jan, L.Y. (1993) Nature 364, 802–806.
[11] Bond, C.T., Ämmälä, C., Ashfield, R., Blair, T.A., Gribble, F., Khan, R.N., Lee, K., Proks, P., Rowe, I.C.M., Sakura, H., Ashford, M.J., Adelman, J.P. and Ashcroft, F.M. (1995) FEBS Lett. 367, 61–66.
[12] Ashford, M.L.J., Bond, C.T., Blair, T.A. and Adelman, J.P. (1994) Nature 370, 456–459.
[14] Bokvist, K., Rorsman, P. and Smith, P.A. (1990) J. Physiol. 423, 327–342.
[15] Thomas, P.M., Cote, G.J., Wohllk, N., Haddad, B., Mathew, P.M., Rabl, W., Aguilar-Bryan, L., Gagel, R.F. and Bryan, J. (1995) Science 268, 425–429.
[16] Dunne, M.J., Kane, C., Squires, P.E., James, R.F.L., Johnson, P.R.V. and Lindley, K.J. (1995) Diabetologia 38, A15.
[17] Yang, J., Jan, Y.N. and Jan, L.Y. (1995) Neuron 14, 1047–1054.