Properties of cloned ATP-sensitive K^+ currents expressed in *Xenopus* oocytes

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1. We have studied the electrophysiological properties of cloned ATP-sensitive K^+ channels (K_{ATP} channels) heterologously expressed in *Xenopus* oocytes. This channel comprises a sulphonylurea receptor subunit (SUR) and an inwardly rectifying K^+ channel subunit (Kir).

2. Oocytes injected with SUR1 and either Kir6.2 or Kir6.1 exhibited large inwardly rectifying K^+ currents when cytosolic ATP levels were lowered by the metabolic inhibitors azide or FCCP. No currents were observed in response to azide in oocytes injected with Kir6.2, Kir6.1 or SUR1 alone, indicating that both the sulphonylurea receptor (SUR1) and an inward rectifier (Kir6.1 or Kir6.2) are needed for functional channel activity.

3. The pharmacological properties of Kir6.2–SUR1 currents resembled those of native β-cell ATP-sensitive K^+ channel currents (K_{ATP} currents): the currents were > 90% blocked by tolbutamide (500 μM), meglitinide (10 μM) or glibenclamide (100 nM), and activated 1·8-fold by diazoxide (340 μM), 1·4-fold by pinacidil (1 mM) and unaffected by cromakalim (0·5 mM).

4. Macroscopic Kir6.2–SUR1 currents in inside-out patches were inhibited by ATP with a K_i of 28 μM. Kir6.1–SUR1 currents ran down within seconds of patch excision preventing analysis of ATP sensitivity.

5. No sensitivity to tolbutamide or metabolic inhibition was observed when SUR1 was coexpressed with either Kir1.1a or Kir2.1, suggesting that these proteins do not couple in *Xenopus* ocytes.

6. Our data demonstrate that the *Xenopus* oocyte constitutes a good expression system for cloned K_{ATP} channels and that expression may be assayed by azide-induced metabolic inhibition.

ATP-sensitive K^+ channels (K_{ATP} channels) couple the metabolic state of the cell to its electrical activity. They thereby play important roles in the physiology and pathophysiology of many tissues, including insulin secretion from pancreatic β-cells, the response to cardiac and cerebral ischaemia and the control of vascular smooth muscle tone (Ashcroft & Rorsman, 1989; Ashcroft & Ashcroft, 1990). A variety of nucleotides, of which ATP and MgADP are of greatest physiological importance, regulate K_{ATP} channel activity. Thus, channel activity is inhibited by ATP with a K_i of ~10 μM (Cook & Hales, 1984) whereas low concentrations of MgADP (100 μM) increase channel activity and relieve the blocking effect of ATP (Kakei, Kelly, Ashcroft & Ashcroft, 1986; Dunne & Petersen, 1986). K_{ATP} channels are also blocked by antidiabetic sulphonylureas such as glibenclamide and tolbutamide, which are used in the treatment of non-insulin-dependent diabetes, and they are activated by K^+ channel openers, such as diazoxide and cromakalim (Ashcroft & Ashcroft, 1992; Dunne, Harding, Jaggar, Ayton & Squires, 1993). The sensitivity to both sulphonylureas and K^+ channel openers varies widely between different tissues.

The β-cell ATP-sensitive K^+ channel comprises a complex of (at least) two proteins: the sulphonylurea receptor, SUR1, and an inwardly rectifying K^+ channel subunit, Kir6.2 (Inagaki *et al.* 1995*a*; Sakura, Ämmälä, Smith, Gribble & Ashcroft, 1995). The sulphonylurea receptor belongs to the ATP-binding cassette (ABC) transporter family (Aguilar-Bryan *et al.* 1995). Like other members of this family, such as the cystic fibrosis transmembrane conductance regulator (CFTR; Higgins, 1992, 1995), the putative topology of SUR1 consists of two groups of transmembrane domains and two nucleotide-binding domains which are located intracellularly. SUR1 acts as the receptor for sulphonylurea drugs (Aguilar-Bryan *et al.* 1995) and may also be required for sensitivity to K^+ channel openers (Ämmälä, Moorhouse & Ashcroft, 1996*a*; Inagaki *et al.* 1996) and to ATP (Inagaki

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et al. 1996; Ämmälä *et al.* 1996*a*). A related sulphonylurea receptor, SUR2, exhibits different pharmacology and ATP sensitivity (Inagaki *et al.* 1996). Kir6.2 is a protein of 391 amino acids and by analogy with what is found for other inward rectifier subunits it seems likely that four Kir6.2 subunits come together to form part of the channel pore. High levels of Kir6.2 mRNA are found in β-cells, skeletal muscle, heart and brain.

Neither Kir6.2 nor SUR1 alone produces functional currents when heterologously expressed in *Xenopus* oocytes or mammalian cell lines (Aguilar-Bryan *et al.* 1995; Inagaki *et al.* 1995*a*; Sakura *et al.* 1995). However, cotransfection of SUR1 and Kir6.2 in HEK293 or Cos cells results in the expression of ATP-sensitive K^+ channel currents (K_{ATP} currents). These currents show pharmacological properties similar to those of native K_{ATP} channels, being inhibited by tolbutamide and activated by diazoxide. In HEK293 cells, whole-cell K_{ATP} currents are initially small but increase more than 4-fold if the cell is dialysed with a low ATP concentration, because the washout of endogenous intracellular ATP removes the resting channel inhibition (Sakura *et al.* 1995).

Early reports indicated that whole-cell currents recorded from *Xenopus* oocytes coinjected with both Kir6.2 and SUR1 mRNAs were of similar amplitude to those of control oocytes (Inagaki *et al.* 1995*a*). However, unlike control oocytes, diazoxide was able to produce a small increase in current amplitude in some coinjected oocytes, an effect which was reversed by tolbutamide. This result suggests that *Xenopus* oocytes may express functional Kir6.2–SUR1 currents but that these are blocked by endogenous ATP levels. Native K_{ATP} channels in many cells are also often totally or partially inhibited by resting concentrations of ATP, but may be activated by metabolic inhibitors such as azide, rotenone, cyanide and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (Ashcroft & Ashcroft, 1990). We therefore examined the effect of decreasing intracellular ATP levels in oocytes coinjected with Kir6.2 and SUR1 mRNAs.

Kir6.1 is an inwardly rectifying K^+ channel with 72% amino acid identity to Kir6.2 (Inagaki *et al.* 1995*b*). It is strongly expressed in heart, ovary and adrenal gland, and at moderate levels in skeletal muscle, lung, brain, stomach, colon, testis and thyroid. K_{ATP} channels sensitive to diazoxide have also been identified in some of these tissues (heart, brain, skeletal muscle and colon; Ashcroft & Ashcroft, 1990). Unlike Kir6.2, Kir6.1 is capable of independent expression in HEK293 cells (Ämmälä *et al.* 1996*a*,*b*). It is not sensitive to either sulphonylureas or to diazoxide when expressed alone, but sensitivity to both these drugs is conferred by coexpression with SUR1. We now report that in oocytes, Kir6.1 does not express independently but that when Kir6.1 is coexpressed with SUR1, large sulphonylurea-sensitive currents are activated by metabolic inhibition.

METHODS

Nomenclature

In this paper we use the nomenclature of Duopnik, Davidson & Lester (1995). Kir6.1 refers to uKATP (Genbank accession number D42145) and Kir6.2 refers to BIR1 (Genbank accession number D50581). Kir6.2 was cloned from a mouse insulinoma cDNA library (Sakura *et al.* 1995). Kir6.1 was cloned from rat pancreatic islets (Inagaki *et al.* 1995*b*) and SUR1 was cloned from hamster insulinoma cells (HIT-T15; Genbank accession number L40623) (Aguilar-Bryan *et al.* 1995).

Molecular biology

Construction of FLAG-tagged Kir6.2. A polymerase chain reaction (PCR) fragment of approximately 300 bp was generated from mouse Kir6.2 cDNA, containing the C-terminal coding sequence with the eight-amino acid 'FLAG' epitope (IBI Kodak) inserted immediately before the stop codon. The PCR fragment was cut with *Eco*47III and *Xba*I, and cloned into Kir6.2 in the pBK-CMV vector (Sakura *et al.* 1995) cut with the same enzymes, so that the PCR fragment replaced the wild-type C-terminal coding region.

***In vitro* transcription.** Kir6.2, Kir6.1, Kir1.1a, Kir2.1, Kir3.1 and SUR1 were cloned into the vectors pBK-CMV, pGEM3Z, pSPORT1, pBF, pBluescript and pcDNA3, respectively. Capped mRNA was synthesized by *in vitro* transcription from linearized cDNA and stored in 10 mM Tris–HCl (pH 7·4) at −80 °C.

Biochemistry

ATP concentration measurements. Batches of five oocytes were incubated in the absence of inhibitor, or in the presence of 3 mM sodium azide or 1 μM FCCP for 0, 10 and 30 min. They were then homogenized in 200 μl of phosphate-buffered saline (PBS; 10 mM phosphate, 137 mM NaCl; pH 7·4). Perchloric acid (100 μl of a 10% solution) was added and the tubes microfuged briefly to pellet cell debris. A 20 μl aliquot of the supernatant was added to 1 ml of ATP buffer (125 mM TrisCl, 5 mM $MgSO_4$, 0·5 mM EDTA, 0·5 mM dithiothreitol, 100 μg ml^{-1} bovine serum albumin; pH 7·8). The ATP content was quantified by the addition of 50 μl luciferin–luciferase extract (firefly lantern extract; Sigma), followed by the measurement of luminescence after 30 s; values obtained were compared with a standard [ATP] curve.

Western blotting. Total oocyte membranes were prepared as described by Tucker, Bond, Herson, Pessia & Adelman (1996). Briefly, twenty-five oocytes were suspended in 1 ml PBS (50 mM phosphate, 150 mM NaCl; pH 8·0) containing protease inhibitors (1 mM each of phenylmethyl-sulphonylfluoride, iodoacetamide and benzamide; and 5 μg ml^{-1} each of leupeptin, aprotinin and pepstatin A) and homogenized by several passages through a 19 gauge needle and two passages through a 25 gauge needle. Homogenates were centrifuged four times at 1000 *g* and 4 °C for 10 min, to remove all yolk granules and melanosomes. The supernatant was then pelleted at 165000 *g* for 30 min to give a total membrane fraction, which was resuspended in 25 μl PBS.

Oocyte plasma membranes were prepared using the method described by Wall & Patel (1989). Briefly, 100 oocytes were broken open using gentle pipetting in cold Hepes buffer (0·25 M sucrose, 10 mM Hepes (pH 7·4), 1 mM EGTA, 2 mM $MgCl_2$ and the protease inhibitors listed above). Plasma membrane complexes (PMCs) were allowed to settle to the bottom of the tube and were removed into 2–4 ml Hepes on ice; they were agitated by repeated pipetting and allowed to settle (for up to 30 min). Several more washes were

performed, in the same tube, to give white PMCs devoid of pigment granules and other organelles. PMCs were transferred into 2 mM $MgCl_2$ (containing protease inhibitors as above) and allowed to settle. PMCs were microfuged for 10 min to concentrate them.

Aliquots of membrane preparations (total or plasma) were subjected to SDS–PAGE using a 9% resolving gel in a BioRad Miniprotean II apparatus. Proteins were Western blotted onto Immobilon-P membrane (Millipore) using an Atto semi-dry Horizblot apparatus (Genetic Research Instumentation, Dunmow, UK). Blots were probed with the m2-FLAG monoclonal antibody (5 μg ml^{-1}; IBI Kodak), overnight at 4 °C (in PBS supplemented with 0·05% Tween 20 and 5% non-fat milk powder). Antibodies were detected using anti-mouse immunoglobulin G antibody coupled to alkaline phosphatase (Sigma).

Electrophysiology

Oocyte collection. Female *Xenopus laevis* frogs were anaesthetized with 3-aminobenzoic acid ethyl ester (Sigma) (2 g l^{-1} added to the water). One ovary was removed via a mini-laparotomy, the incision sutured and the animal allowed to recover. Once the wound had completely healed, the second ovary was removed in a similar operation and the animal was then killed by decapitation whilst under anaesthesia.

Oocyte preparation. Immature stage V–VI *Xenopus* oocytes were incubated for 75 min with 1·5 mg ml^{-1} collagenase (Type A; Boehringer) and manually defolliculated. Oocytes were then coinjected with a single type of mRNA or a mixture of mRNAs, as indicated. Unless otherwise indicated, equal amounts (~25 ng) of the different mRNAs were mixed prior to injection. Control oocytes were injected with 10 mM Tris–HCl. The final injection volume was ~50 nl per oocyte in all cases. Isolated oocytes were maintained in modified Barth's solution containing (mM): 88 NaCl, 1 KCl, 1·7 $MgSO_4$, 0·47 $Ca(NO_3)_2$, 0·41 $CaCl_2$, 2·4 $NaHCO_3$, 10 Hepes (pH 7·4), supplemented with 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 5 mM pyruvate. Currents were studied 1–4 days after injection.

Two-electrode voltage clamp. Whole-cell currents were measured using a two-electrode voltage-clamp (Geneclamp 500; Axon Instruments). Voltages were applied and currents recorded using a microcomputer with an AxoLab interface and pCLAMP software (Axon Instruments). Currents were filtered at 1 kHz and digitized at 4 kHz. Current and voltage electrodes were filled with 3 M KCl and had resistances of 0·5–2 MΩ. Transmembrane potential was measured differentially between the intracellular electrode and a second bath electrode positioned close to the oocyte on the downstream side, in order to minimize series resistance errors. The bath electrodes consisted of Ag–$AgCl_2$ pellets connected to the bath by agar bridges filled with 3 M KCl. Recordings were initiated in a solution containing (mM): 87·5 NaCl, 2·5 KCl, 1 $MgCl_2$, 1·8 $CaCl_2$, 5 Hepes (pH 7·4 with NaOH) and the solution was then replaced by a high-potassium (90 mM K^+) bath solution containing (mM): 90 KCl, 1 $MgCl_2$, 1·8 $CaCl_2$, 5 Hepes (pH 7·4 with KOH). Diazoxide was prepared from a stock solution (68 mM) in water as required. Sodium azide was prepared as a stock solution (1000 ×) in water. Tolbutamide, glibenclamide, meglitinide and cromakalim were prepared as 1000 × stock solutions in DMSO and diluted as required. Pinacidil was prepared as a 100 × stock solution in ethanol. $BaCl_2$ was added directly to the solution. Oocytes were continuously perfused with the solutions and the experiments carried out at room temperature (18–24 °C). Whole-cell currents were measured 280–295 ms after the start of the voltage pulse.

Table 1. Effects of metabolic inhibition on $[ATP]_i$

	$[ATP]_i$ (pmol ATP per oocyte)		
	10 min	20 min	60 min
Control	1170	—	—
Azide	850	850	1010
FCCP	990	860	900

Each value is the mean of 5 samples, each consisting of 5 oocytes (i.e. $n = 25$). Times of incubation with inhibitors are indicated.

Giant patch recordings. Macroscopic currents were recorded at 20–24 °C from giant inside-out patches (Hilgemann, Nicoll & Phillipson, 1991) excised from oocytes coinjected with Kir6.2 and SUR1, using 200–400 kΩ electrodes. The pipette solution contained (mM): 140 KCl, 1·2 $MgCl_2$, 2·6 $CaCl_2$, 10 Hepes (pH 7·4 with KOH) and the internal (bath) solution contained (mM): 110 KCl, 1·44 $MgCl_2$, 30 KOH, 10 EGTA, 10 Hepes (pH 7·2 with KOH). MgATP was rapidly applied by positioning the patch in the mouth of one of a series of adjacent inflow pipes placed in the bath. Currents were recorded with an EPC7 amplifier (List Electronik, Darmstadt, Germany), filtered at 0·2 kHz and sampled at 0·5 kHz.

Data analysis

All data are given as means ± S.E.M. The symbols in the figures indicate the means, and the vertical bars, where larger than the symbol, indicate 1 S.E.M. Control currents in the absence of azide have not been subtracted.

RESULTS

Injection of oocytes with mRNA encoding SUR1, Kir6.2, or SUR1 and Kir6.2 together, did not result in resting currents which were different from those of control oocytes. In oocytes injected with either Kir6.2 or SUR1 mRNA alone, no change in current amplitude was observed when the metabolic inhibitor azide (3 mM) was added to the bath solution (Fig. 1*A* and *B*). By contrast, azide produced a gradual increase in whole-cell currents in oocytes coinjected with Kir6.2 and SUR1, which began 3–5 min after azide application and reached a steady-state approximately 10 min later (Fig. 1*C*). The effect of azide was reversible on washout of the inhibitor. However, as shown in Fig. 1*C*, there was an initial transient increase in current following azide removal, which suggests that some product of metabolic inhibition, or azide itself, may directly block Kir6.2–SUR1 currents.

The simplest explanation of these results is that azide produces a fall in intracellular ATP and thereby activates an ATP-sensitive K^+ current formed by Kir6.2–SUR1. To confirm this hypothesis, we tested the effect of another metabolic inhibitor with a different site of action. Azide inhibits the mitochondrial electron transport protein cytochrome a_3 (Tsubaki & Yoshikawa, 1993) and the F_1/F_0

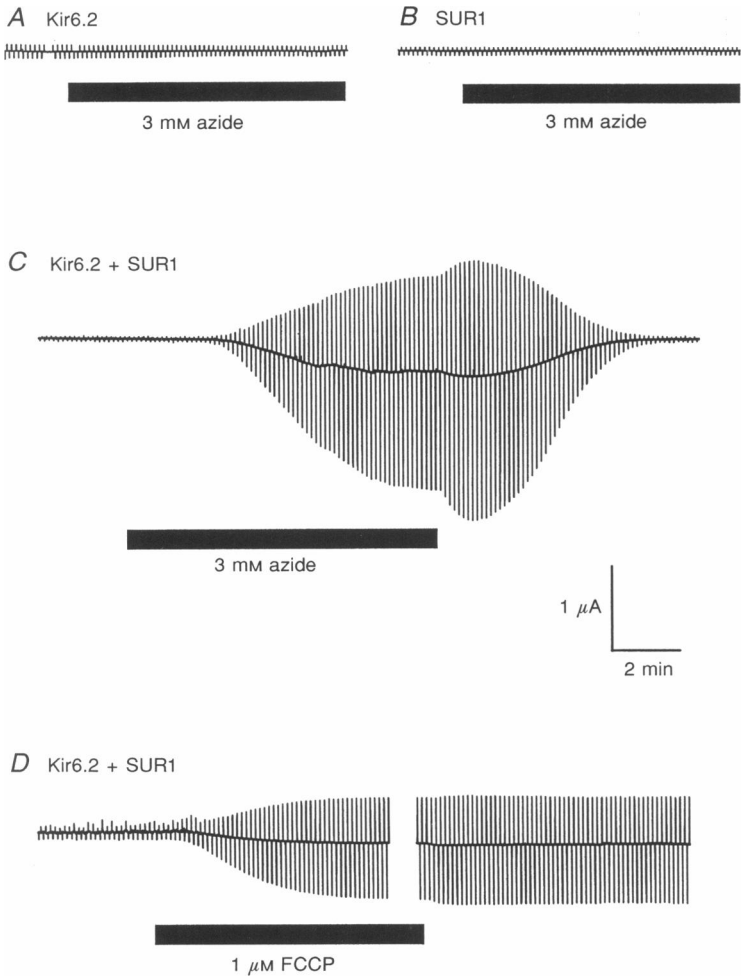

Figure 1. Metabolic inhibition activates whole-cell K_{ATP} currents

Whole-cell currents recorded in response to alternate 20 mV depolarizing or hyperpolarizing pulses from a holding potential of −10 mV in oocytes injected with mRNA encoding Kir6.2 (*A*) or SUR1 (*B*) alone or both SUR1 and Kir6.2 (*C* and *D*). Azide (3 mM) or FCCP (1 μM) were added to the bath solution as indicated by the horizontal bars.

ATPase (Vasilyeva, Minkov, Fitin & Vinogradov, 1982), whereas the proton ionophore FCCP uncouples mitochondrial electron transport from ATP synthesis. FCCP (1 μM) also activated a K^+ current with a delay of 3–5 min, but in this case the effect of the inhibitor could not be reversed by 10 min washing (Fig. 1*D*). The mean current amplitude at −100 mV was 0·30 ± 0·04 μA before, and 4·5 ± 1·2 μA ($n = 6$) 10 min after, application of FCCP. This is not significantly different from the increase in current produced by azide in the same batch of oocytes (from 0·22 ± 0·03 to 5·8 ± 1·8 μA, $n = 5$, in the absence and presence of azide, respectively). There was no effect of FCCP on control oocytes, the mean current at −100 mV being 0·15 ± 0·01 μA before and 0·20 ± 0·03 μA ($n = 5$) after FCCP. In the remainder of this paper, we have used azide in all electrophysiological studies, as the effects of FCCP were not reversible.

We measured the effects of metabolic inhibition on ATP levels in uninjected oocytes. As shown in Table 1, both azide and FCCP caused a significant fall in ATP levels within 10 min. Assuming a volume of 500 nl (corresponding to an average oocyte diameter of 1 mm), this corresponds to a decrease in total $[ATP]_i$ from 2·3 to 1·7 mM after 10 min exposure to azide. It should be emphasized that these values

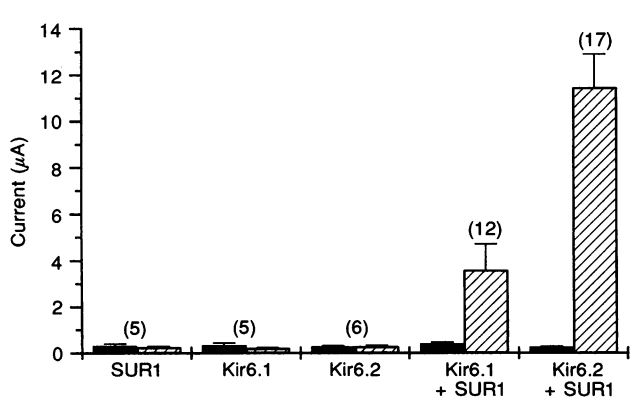

Figure 2. Histogram of whole-cell currents induced by 3 mM azide

Mean whole-cell currents recorded at −100 mV in the absence (■) and presence (▨) of 3 mM azide from oocytes injected with the mRNA(s) indicated. The number of cells is given in parentheses above the bars.

represent the total ATP concentration averaged over the whole oocyte and not that directly beneath the plasma membrane.

Figure 2 illustrates the mean current amplitudes before and after azide application for oocytes injected with various mRNAs. Whole-cell currents in oocytes injected with Kir6.2, Kir6.1 and SUR1 alone were of similar size to those of control oocytes and were unaffected by azide. When oocytes were coinjected with SUR1 and either Kir6.1 or 6.2 mRNAs, resting currents were of similar size to those observed in control oocytes, but azide now induced a marked activation: this amounted to an 8·6 ± 2·1-fold ($n = 12$) increase in current with Kir6.1 + SUR1 and a 45·5 ± 4·5-fold ($n = 17$) increase for Kir6.2 + SUR1.

Figure 3*A* shows whole-cell currents (*a* and *b*) and corresponding *I–V* relations (*c*) recorded from two different

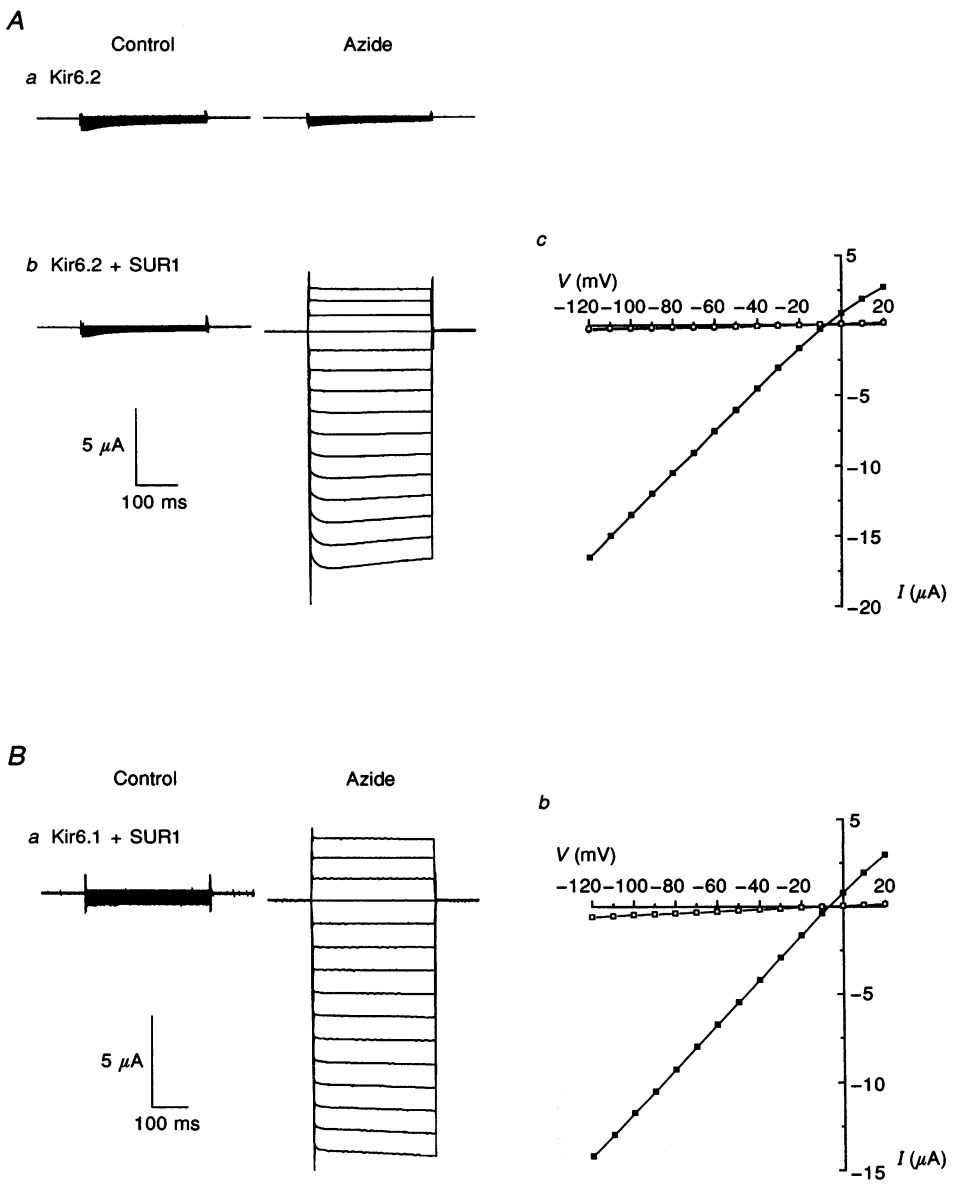

Figure 3. Whole-cell *I–V* relations for Kir6.2–SUR1 and Kir6.1–SUR1

A a and *b*, whole-cell currents recorded from two different oocytes injected with mRNA encoding Kir6.2 (*a*) or Kir6.2 + SUR1 (*b*) in control solution (left) and 10 min after exposure to 3 mM azide (right). Currents were elicited by a series of voltage steps from −120 to +20 mV from a holding potential of −10 mV. *c*, corresponding *I–V* relationships recorded in the absence (open symbols) and presence (filled symbols) of 3 mM azide for the same oocytes as in *a* and *b*: ○ and ●, Kir6.2; □ and ■, Kir6.2 + SUR1. The symbols ○, ● and □ all overlap. *B a*, whole-cell currents recorded from an oocyte injected with mRNA encoding Kir6.1 + SUR1 in control solution (left) and 10 min after exposure to 3 mM azide (right). Currents were elicited by a series of voltage steps from −120 to +20 mV from a holding potential of −10 mV. *b*, corresponding *I–V* relationships recorded in the absence (□) and presence (■) of 3 mM azide for the same oocyte as in *B a*.

oocytes injected with Kir6.2, or Kir6.2 + SUR1 mRNAs in the absence and subsequently the presence of azide. Azide induced a large, weakly inwardly rectifying current in oocytes coinjected with Kir6.2 + SUR1 but not in those injected with Kir6.2 alone. Similar results were observed with Kir6.1 + SUR1 (Fig. 3*B*). The rectification of Kir6.2–SUR1 currents was somewhat greater than that observed when the channel is heterologously expressed in HEK293 cells (Sakura *et al.* 1995). Furthermore, whole-cell Kir6.2–SUR1 currents showed a time-dependent activation on hyperpolarization (Fig. 3*A*): in contrast, Kir6.2–SUR1 currents expressed in HEK293 cells activate instantaneously on hyperpolarization (Sakura *et al.* 1995), as is also the case for the native β-cell K_{ATP} channel (Takano & Ashcroft, 1996).

The reversal potential of Kir6.2–SUR1 currents was $-4{\cdot}1 \pm 0{\cdot}4$ mV ($n = 18$) in 90 mM external K^+. Decreasing external potassium (Na^+ replacement) decreased the slope conductance and shifted the reversal potential to more negative potentials (Fig. 4). The mean change in the reversal potential was $56{\cdot}7 \pm 0{\cdot}2$ mV ($n = 4$) for a 10-fold change in $[K^+]_o$. This indicates that Kir6.2–SUR1 currents are highly K^+ selective. The relative permeability to Na^+ was calculated from the Goldman–Hodgkin–Katz equation and gave a permeability ratio (P_{Na}/P_K) of 0·008. This is in good agreement with that found for the native β-cell K_{ATP} channel ($P_{Na}/P_K = 0{\cdot}007$; Ashcroft, Kakei & Kelly, 1989).

The pharmacology of the whole-cell Kir6.2–SUR1 current activated by azide was consistent with that expected for the native β-cell K_{ATP} channel. Thus, whole-cell currents were almost completely blocked by 0·5 mM tolbutamide (Fig. 5), 100 nM glibenclamide, 10 μM meglitinide or 5 mM barium (Table 2A). Tolbutamide was without effect in control oocytes, or in Kir6.2–SUR1-injected oocytes in the absence of azide: this indicates that whole-cell K_{ATP} currents are completely blocked by resting ATP concentrations in the oocyte. The currents activated by FCCP were also largely abolished by 0·5 mM tolbutamide ($88{\cdot}2 \pm 1{\cdot}2$% block). Inhibition of azide-induced currents by tolbutamide, glibenclamide and meglitinide was not reversible within 10 min of washing, a finding which probably results from accumulation of the lipid-soluble drug within the oocyte.

Table 2. Effects of drugs on whole-cell Kir6.2–SUR1 currents

A.	Drug	Concentration	% Block	*n*
	Tolbutamide	500 μM	$96{\cdot}0 \pm 0{\cdot}5$	5
	Meglitinide	10 μM	$94{\cdot}4 \pm 1{\cdot}2$	5
	Glibenclamide	100 nM	$94{\cdot}9 \pm 0{\cdot}8$	5
	Barium	5 mM	$97{\cdot}8 \pm 0{\cdot}2$	6
B.	Drug	Concentration	% Activation	*n*
	Diazoxide	340 μM	179 ± 2	7
	Pinacidil	1 mM	139 ± 6	5
	Cromakalim	0·5 mM	105 ± 2	5

The effects of all drugs were tested in the presence of 3 mM azide.

The effects of K^+ channel openers are shown in Table 2B. Diazoxide (340 μM) potentiated azide-activated currents by ~2-fold (see also Fig. 5), pinacidil (1 mM) produced a small activation and cromakalin (0·5 mM) was without effect. This sensitivity is similar to that found for the native β-cell K_{ATP} channel. Diazoxide also enhanced the currents in the absence of azide: the currents increased from $0{\cdot}35 \pm 0{\cdot}11$ to $2{\cdot}45 \pm 0{\cdot}17$ μA ($n = 4$). No effects of ethanol or DMSO, in

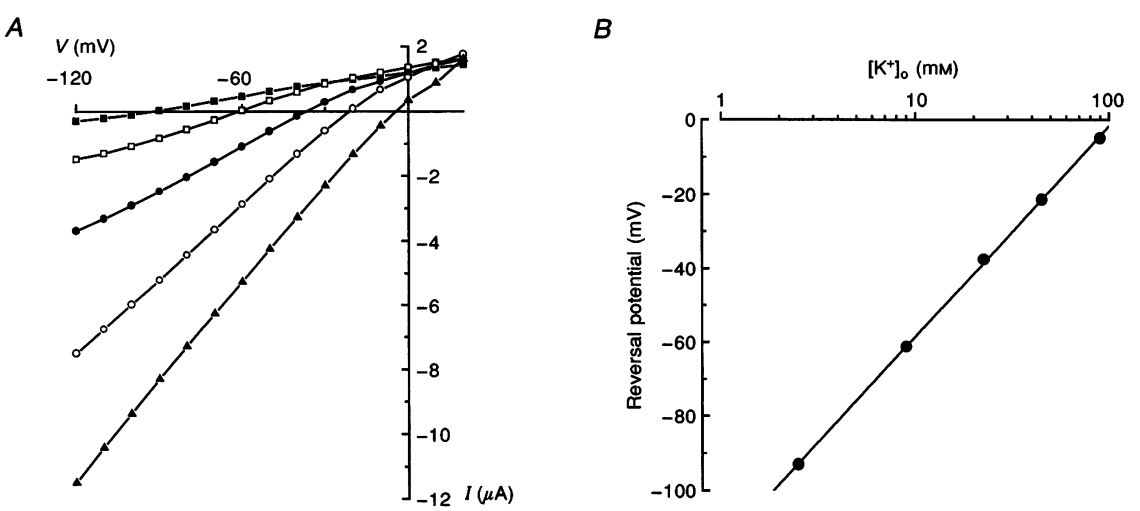

Figure 4. Effect of external potassium on whole-cell K_{ATP} currents

A, *I–V* relationships recorded from the same oocyte in solutions with the following K^+ concentrations (mM): 90 (▲), 45 (○), 22·5 (●), 9 (□) and 2·5 (■). All solutions also contained 3 mM azide. The holding potential was the zero current potential in each solution. The oocyte was injected with Kir6.2 and SUR1 mRNAs. *B*, mean relationship between the current reversal potential and $[K^+]_o$ for 4 cells. The line has a slope of 56·7 mV per decade change in $[K^+]_o$; S.E.M. values are smaller than the symbols.

which the drugs were dissolved, were observed. Likewise, diazoxide was without effect on uninjected oocytes.

To confirm that Kir6.2–SUR1 currents were ATP sensitive, we measured the dose–response curve for ATP in inside-out giant patches excised from oocytes coinjected with Kir6.2 and SUR1. Prior to excision, the patch conductance was very low, but it increased rapidly following excision (Fig. 6*A*). The mean patch conductance measured between −110 and +40 mV in five patches was 0·13 ± 0·02 nS before and 15·58 ± 5·43 nS after patch excision. This increase in conductance was not observed in oocytes injected with Tris, indicating that it results from activation of Kir6.2–SUR1 currents which are inhibited in the cell-attached configuration. In inside-out patches, there was no time-dependent activation of Kir6.2–SUR1 currents on hyperpolarization and the rectification of the *I–V* relation was less pronounced than that observed for whole-cell currents (Fig. 6*B* and *C*). The ratio of the currents recorded at +25 mV from the reversal potential to those recorded at −25 mV from the reversal potential was 0·67 ± 0·07 mV ($n = 7$) for whole-cell currents and 0·91 ± 0·01 mV ($n = 5$; $P < 0·02$) for macroscopic currents in inside-out patches. These results suggest that both the time-dependent activation and the rectification of whole-cell currents are conferred by some cytoplasmic constituent which is lost on patch excision.

Application of ATP to the intracellular membrane surface produced a reversible, concentration-dependent inhibition of the macroscopic Kir6.2–SUR1 currents (Fig. 7*A*). Like the native K_{ATP} channel (Ashcroft & Rorsman, 1989), this block was independent of membrane potential. Following washout of 1 mM ATP, the conductance was greater than that observed before ATP application (6·5 ± 2·2 compared with 4·9 ± 2·0 nS; $n = 5$, $P < 0·05$). This phenomenon, known as refreshment or reactivation, is also observed for native K_{ATP} channels (Ashcroft & Ashcroft, 1990). The mean dose–response curve for ATP inhibition of Kir6.2–SUR1 currents is given in Fig. 7*B*, and was fitted to the Hill equation. Under control conditions, mean values for the Hill coefficient (n_H) were 1·0 ± 0·1 ($n = 5$) and the ATP concentration which produced half-maximal inhibition (K_i) was 28·3 ± 4·5 μM ($n = 5$). These values are in reasonable agreement with those observed for native K_{ATP} channels (Ashcroft & Rorsman, 1989).

After initially increasing on patch excision, Kir6.2–SUR1 currents declined with time. The rate of this decline was very variable: in general, an initial rapid decline was followed by sustained level of current which ran down more slowly. By contrast, Kir6.1–SUR1 currents ran down so rapidly on patch excision (within 10 s) that it was not possible to test the ATP sensitivity, or in most cases, to obtain a full current–voltage relation.

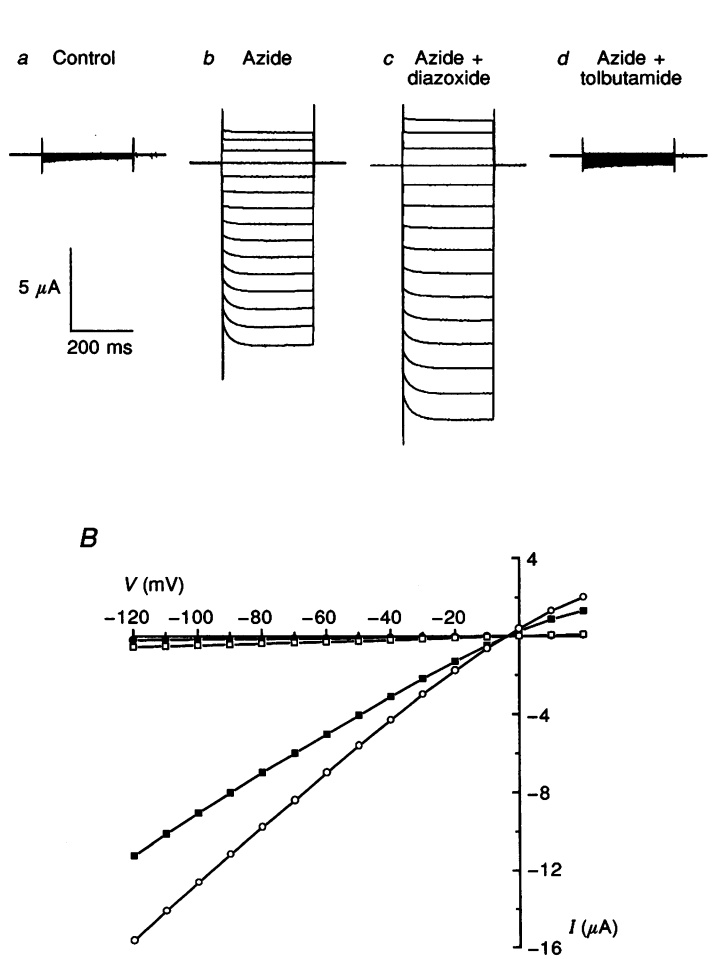

Figure 5. Effects of diazoxide and tolbutamide on whole-cell K_{ATP} currents
A, whole-cell currents recorded consecutively from an oocyte coinjected with mRNA encoding Kir6.2 and SUR1 in control solution (*a*), in 3 mM azide (*b*), in 3 mM azide + 340 μM diazoxide (*c*) and in 3 mM azide + 0·5 mM tolbutamide (*d*). *B*, *I–V* relationships recorded for the same oocyte in control solution (●), then in 3 mM azide (■), subsequently in 3 mM azide + 340 μM diazoxide (○) and finally in 3 mM azide + 0·5 mM tolbutamide (□).

Table 3. Effects of metabolic inhibition and drugs on Kir channels coexpressed with SUR1

Clone	I (μA) Control	I (μA) Azide	% Block Tolbutamide	n
Water	0·3 ± 0·1	0·24 ± 0·05	n.t.	5
Kir1.1a	11·6 ± 2·2	11·4 ± 1·9	2·7 ± 1·5	5
Kir2.1	4·4 ± 1·1	4·8 ± 1·5	−2·8 ± 2·4	5
Kir3.1	0·39 ± 0·05	0·31 ± 0·04	−2·7 ± 7·6	5
Kir6.1	0·39 ± 0·07	3·60 ± 1·11	75·3 ± 8·1*	12
Kir6.2	0·26 ± 0·03	11·5 ± 1·5	96·0 ± 0·5†	17
fKir6.2	0·29 ± 0·09	32·9 ± 12·5	97·2 ± 0·6	4

* $n = 8$; † $n = 5$; n.t., not tested. Currents were measured at −100 mV in 90 mM K^+ solution (Control) then in the presence of 3 mM azide and finally 3 mM azide + 0·5 mM tolbutamide. The amount of mRNA injected per oocyte was: Kir1.1a, 1 ng; Kir2.1, 0·5 ng; Kir3.1, 25 ng; Kir6.1, 25 ng; Kir6.2, 25 ng; and fKir6.2, 5–25 ng.

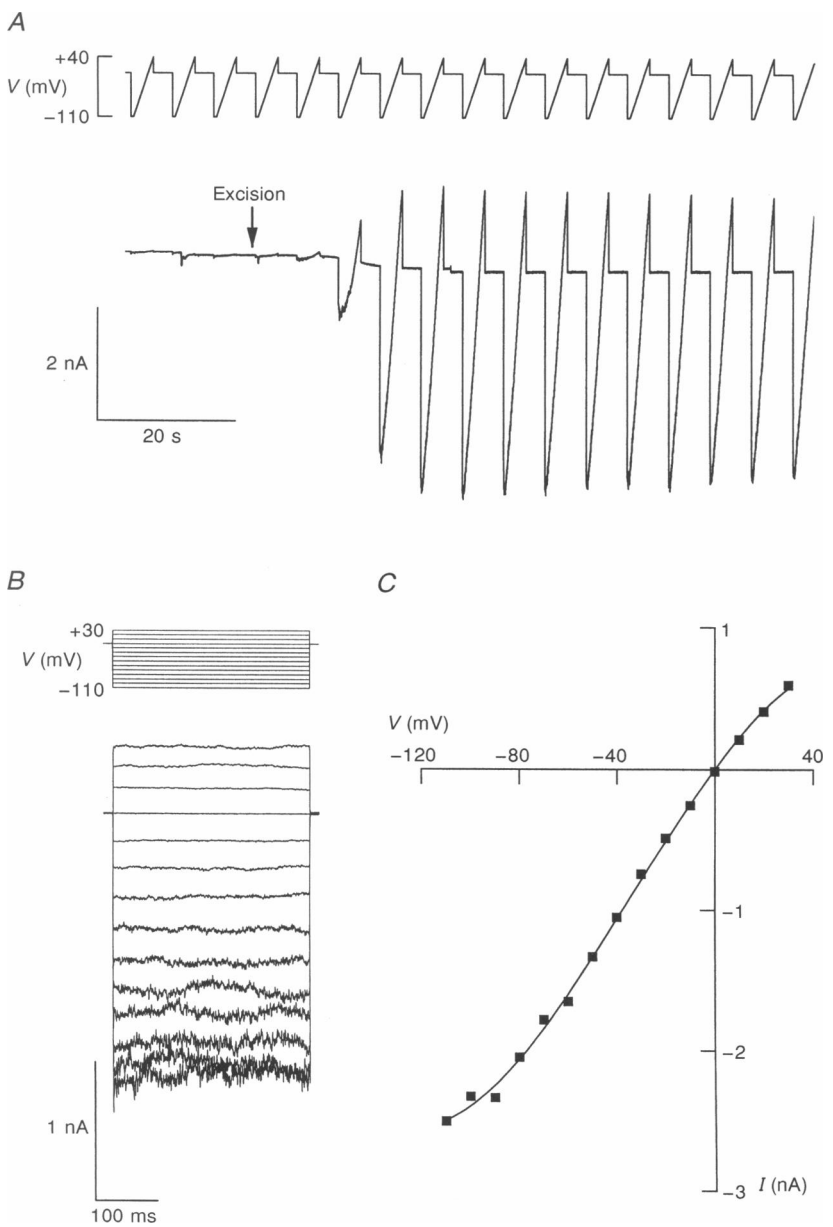

Figure 6. Macroscopic K_{ATP} currents in inside-out membrane patches

A, macroscopic currents recorded from a giant inside-out patch excised from an oocyte coinjected with Kir6.2 and SUR1. The pulse protocol is shown above: the holding potential was 0 mV and the voltage was successively ramped from −110 to +40 mV over a 5 s period. The patch was excised at the arrow into ATP-free solution. *B*, whole-cell currents (below) elicited by a series of depolarizations from −110 to +30 mV from a holding potential of 0 mV (above). *C*, *I–V* relation for the same patch as in *B*. Note that azide was not present in these experiments.

We also examined the effects of coexpression of SUR1 with the related inward rectifiers Kir1.1a (ROMK1), Kir2.1 (IRK1) and Kir3.1 (GIRK1) in whole-cell studies (see Doupnik *et al.* 1995 for terminology of inward rectifiers). Table 3 shows that there was no effect of either azide or tolbutamide on these currents. This indicates that, in *Xenopus* oocytes, SUR1 neither forms functional channels with Kir3.1 (as it does with Kir6.2 and Kir6.1), nor endows Kir1.1a or Kir2.1 with either ATP or sulphonylurea sensitivity.

To examine whether Kir6.2 requires SUR1 for stable expression in oocyte membranes, we placed an eight-amino acid epitope tag (FLAG) on the C-terminus of Kir6.2 cDNA. Coexpression of the FLAG-tagged Kir6.2 (fKir6.2) with SUR1 resulted in whole-cell currents that were induced by azide, inhibited by tolbutamide and activated by diazoxide (Table 3). This indicates that the epitope tag does not impede the interaction between Kir6.2 and SUR1 and further suggests that a free C-terminus of Kir6.2 is not critical for this interaction. Total membranes were prepared from oocytes expressing fKir6.2 and probed with a monoclonal antibody to the epitope tag of fKir6.2. As shown in Fig. 8, a protein of apparent molecular mass of 38 kDa was labelled in membranes from oocytes injected with fKir6.2 or fKir6.2 + SUR1, but not from oocytes injected with SUR1 mRNA alone. This is in reasonable agreement with the predicted molecular mass of 43 kDa for Kir6.2 (the difference reflects the fact that proteins may run aberrantly on SDS gels). Thus these data indicate that Kir6.2 is translated, and stably targeted to the membrane in oocytes even in the absence of SUR1. It is likely that most of the membranes in our total membrane preparation derive from intracellular membrane systems such as the endoplasmic reticulum. Thus these studies of total membranes do not address the question of whether Kir6.2 may reach the surface (plasma) membrane in the absence of SUR1, or whether it is retained within the internal membrane system. In an attempt to address this question, we prepared purified plasma membranes from oocytes injected with fKir6.2 + SUR1 or Kir6.2 alone (data not shown). Unfortunately, we were unable to detect Kir6.2 even in control plasma membranes (fKir6.2 + SUR1), presumably because the protein concentration was too low. Thus we were unable to examine whether fKir6.2 requires SUR1 for targeting to the plasma membrane.

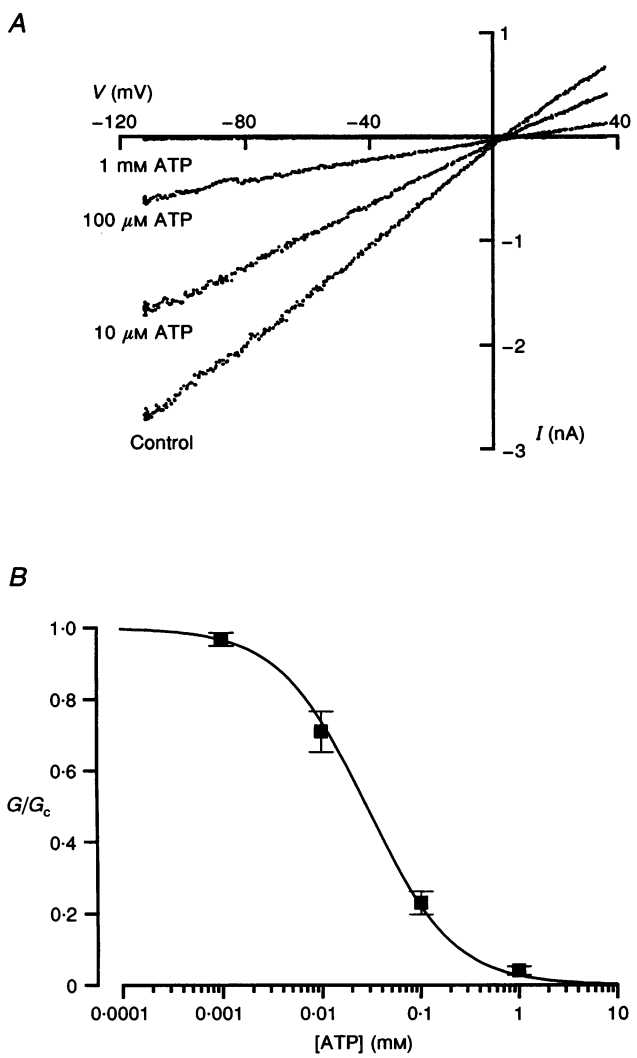

Figure 7. ATP inhibition of macroscopic K_{ATP} currents in inside-out patches

A, Kir6.2–SUR1 *I–V* relations obtained from an inside-out patch at different ATP concentrations. Three consecutive ramps have been averaged for each ATP concentration. *B*, mean ATP dose–response curves for Kir6.2–SUR1 currents recorded in the inside-out patch configuration ($n = 5$ oocytes). Test solutions were alternated with control solutions and the conductance (G) was expressed as a fraction of the mean (G_c) of that obtained in control solution before and after exposure to ATP. The slope conductance was measured by fitting a straight line to the ramped *I–V* relation between −20 and −100 mV. Three *I–V* values were averaged for each ATP concentration. In each case, the ATP dose–response curve was fitted to the Hill equation:

$$\frac{G}{G_c} = \frac{1}{1 + ([\mathrm{ATP}]/K_i)^{n_H}}, \qquad (1)$$

where [ATP] is the ATP concentration, K_i is the ATP concentration at which inhibition is half-maximal and n_H is the slope factor (Hill coefficient). The line is drawn to eqn (1) using the mean values obtained for K_i (28·3 μM) and n_H (1). Note that azide was not present in these experiments.

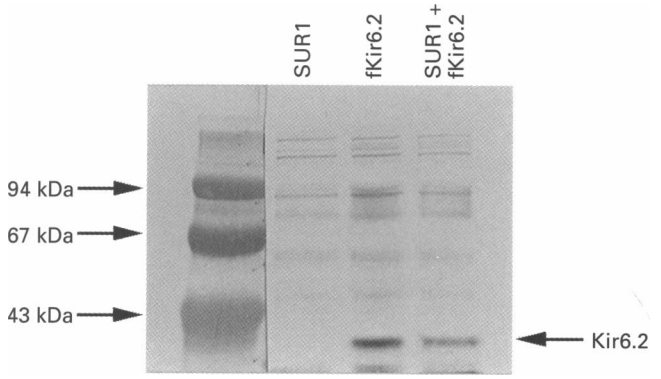

Figure 8. Western blot of SUR1, Kir6.2 and Kir6.2 + SUR1

Western blot of total membranes prepared from oocytes injected with SUR1, fKir6.2, or fKir6.2 + SUR1, and probed with the FLAG antibody. Molecular weight markers are shown in the left-hand lane. The arrow indicates the position of fKir6.2 (38 kDa).

DISCUSSION

Our results demonstrate that the cloned β-cell K_{ATP} channel complex (Kir6.2–SUR1) expresses functional channel activity in *Xenopus* oocytes. Whole-cell currents are inhibited at resting intracellular ATP concentrations and can only be observed following metabolic inhibition with azide or FCCP, which lowered ATP levels from 2·3 to 1·7 and 2 mM, respectively. These ATP concentrations are considerable higher than those required to produce half-maximal inhibition of Kir6.2–SUR1 (28 μM) or native K_{ATP} (~10 μM; Ashcroft & Ashcroft, 1990) currents in inside-out patches. Indeed, the ATP concentrations measured in the presence of azide would be expected to block the Kir6.2–SUR1 current fully (Fig. 7*B*). A similar phenomenon is found for native β-cell K_{ATP} currents where 0·3 mM ATP fully activates the channel in whole-cell recordings (Trube, Rorsman & Ohno-Shosaku, 1986) and the K_i for ATP inhibition in intact β-cells is around 1·5 mM (Niki, Ashcroft & Ashcroft, 1989). There are at least two explanations for these findings (Ashcroft & Rorsman, 1989). First, the fall in ATP is accompanied by a concomitant rise in MgADP which promotes channel activity and reduces the inhibitory action of ATP. Secondly, the ATP measurements reflect the average ATP concentration of the oocyte and not that directly beneath the plasma membrane. There is evidence for spatial compartmentalization of cytoplasmic ATP in *Rana pipiens* oocytes (Miller & Horowitz, 1986).

We observed a number of differences between whole-cell Kir6.2–SUR1 currents expressed in *Xenopus* oocytes and those reported for HEK293 cells (Sakura *et al.* 1995), including a greater degree of inward rectification and a time-dependent current activation on hyperpolarization. The characteristic rectification of inwardly rectifying K^+ channels results from a voltage-dependent block by intracellular cations such as Mg^{2+} and spermine (Doupnik *et al.* 1995; Shyng, Clement, Bryan & Nichols, 1996). It therefore seems probable that the stronger rectification observed in the present experiments may arise from the presence of higher concentrations of endogenous polyamines and divalent cations in the oocyte cytoplasm, since whole-cell Kir6.2–SUR1 currents were recorded from HEK293 cells dialysed with an intracellular solution containing minimal blocking ions. This idea is supported by the fact that less rectification is observed for I–V relations recorded from inside-out patches exposed to 1·0 mM free Mg^{2+}.

The time-dependent activation of Kir6.2–SUR1 currents in oocytes cannot result from a series resistance error since it did not vary with current amplitude (i.e. level of expression) and was not observed for Kir1.1a currents, which are of similar magnitude. The fact that activation was not observed in inside-out patches suggests it may result from a time-dependent unblock of the current on hyperpolarization, as described for other inward rectifiers (cf. Doupnik *et al.* 1995).

In addition to causing channel activation, azide also appears to inhibit the cloned K_{ATP} channel since an initial transient increase in the whole-cell current was observed when azide was removed. This unblock was very rapid and was observed immediately on removing azide: in some oocytes addition of azide also produced an immediate inhibition of the resting currents before a delayed activation. The rapidity of block, together with the fact that it is not observed with FCCP, suggests that the block may result from an effect of azide itself rather than from some product of metabolic inhibition. Consistent with this idea, azide did inhibit whole-cell K_{ATP} currents in native β-cells dialysed with 0·3 mM ATP (S. Trapp, personal communication). Interestingly, azide has been shown to prevent ATP hydrolysis at the first nucleotide-binding domain of CFTR, another member of the ABC transporter family (Ko & Pedersen, 1995), and the F_1/F_0 ATPase (Vasilyeva *et al.* 1982).

The pharmacology of the cloned K_{ATP} channel resembles that of the native β-cell K_{ATP} channel. Thus, the current is inhibited by the sulphonylureas tolbutamide and glibenclamide, and by the benzamido derivative meglitinide, which corresponds to the non-sulphonylurea moiety of glibenclamide. In β-cells, diazoxide is the most effective of the K^+ channel openers at activating the K_{ATP} channel,

pinacidil has a small effect and cromakalim is without effect. By contrast, cromakalim is an effective opener of the smooth muscle K_{ATP} channel and diazoxide inhibits, rather than activates, the cardiac K_{ATP} channel (Ashcroft & Ashcroft, 1992). The relative potency of K^+ channel openers in activating Kir6.2–SUR1 currents is consistent with that of the native β-cell channel, but not with that of cardiac or smooth muscle and suggests the K_{ATP} channel in these tissues may be comprised of different subunits. This idea has recently been confirmed directly for cardiac K_{ATP} channels, which have been shown to comprise of a complex of Kir6.2 and SUR2 (Inagaki *et al.* 1996).

No azide-activated currents were observed in oocytes injected with Kir6.2 alone. This is consistent with previous observations on Cos cells and HEK293 cells where neither whole-cell nor single-channel K_{ATP} currents were observed following transfection with Kir6.2 (Inagaki *et al.* 1995*a*; Sakura *et al.* 1995). Our data demonstrate that Kir6.2 does not require SUR1 for stable expression in intracellular oocyte membranes, but we were unable to determine whether Kir6.2 is able to enter the plasma membrane in the absence of SUR1. Thus we do not know whether SUR1 is required for targeting of Kir6.2 to the surface membrane or if it is needed for expression of functional channel activity.

Our data suggest that like Kir6.2, Kir6.1 is able to form K_{ATP} channels in conjunction with SUR1. However, SUR1 did not confer sensitivity to sulphonylureas or to metabolic inhibition on subunits from other inward rectifier families such as Kir1.1a, Kir2.1 or Kir3.1.

It is of interest that Kir6.1 did not express alone in *Xenopus* oocytes, in contrast to what has been reported previously by others (Inagaki *et al.* 1995*b*), and by ourselves for HEK293 cells (Ämmälä *et al.* 1996*b*). The reason for these differences is unclear. One possibility is that functional expression of Kir6.1 (in the absence of SUR1) may require additional subunits, or channel modulators, which are endogenously present in HEK293 cells but not in oocytes. In this respect it is of interest that HEK293 cells express an endogenous inward rectifier, and that SUR1 can confer sulphonylurea sensitivity upon this channel (Ämmälä *et al.* 1996*b*). Since related inward rectifiers may form heteromeric channels with novel properties (Doupnik *et al.* 1995), it seems possible that Kir6.1 (but not Kir6.2) can couple to the endogenous Kir channel in HEK293 cells to form a heteromeric channel which is capable of independent expression. A similar argument may be applied to the finding that there was no apparent coupling between ROMK1 and SUR1 in oocytes, although coupling was observed in HEK293 cells (Ämmälä *et al.* 1996*b*). Possibly, only heteromeric channels are able to couple to SUR1.

In conclusion our data indicate that the *Xenopus* oocyte constitutes a good expression system for the cloned β-cell K_{ATP} channel (Kir6.2–SUR1) and that expression may be assayed by azide-induced metabolic inhibition.

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