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Sensitivity of Kir6.2-SUR1 currents, in the absence and presence of sodium azide, to the K_{ATP} channel inhibitors, ciclazindol and englitazone

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1 Two electrode voltage clamp and single channel recordings were used to investigate the actions of various ATP-sensitive K^+ (K_{ATP}) channel inhibitors on cloned K_{ATP} channels, expressed in *Xenopus* oocytes and HEK 293 cells.

2 Oocytes expressing Kir6.2 and SUR1 gave rise to inwardly rectifying K^+ currents following bath application of 3 mM sodium azide. Inside-out recordings from non-azide treated oocytes demonstrated the presence of K_{ATP} channels which were activated by direct application of 3 mM azide and 0.1 mM Mg-ATP.

3 Tolbutamide inhibited azide-induced macroscopic Kir6.2-SUR1 currents, recorded from *Xenopus* oocytes, with an IC_{50} value similar to native K_{ATP} channels. Ciclazindol and englitazone also inhibited these currents in a concentration-dependent manner, but with relative potencies substantially less than for native K_{ATP} channels.

4 Single channel currents recorded from inside-out patches excised from oocytes expressing Kir6.2-SUR1 currents were inhibited by tolbutamide, Mg-ATP, englitazone and ciclazindol, in the absence of azide, with potencies similar to native K_{ATP} channels. In the presence of azide, Kir6.2-SUR1 currents were inhibited by englitazone and tolbutamide but not ciclazindol.

5 Single channel currents derived from Kir6.2Δ26, expressed in HEK 293 cells, were inhibited by ciclazindol and englitazone irrespective of the absence or presence of SUR1.

6 In conclusion, heterologously expressed Kir6.2 and SUR1 recapitulate the pharmacological profile of native pancreatic β-cell K_{ATP} channels. However, currents induced by azide exhibit a substantially reduced sensitivity to ciclazindol. It is likely that ciclazindol and englitazone inhibit K_{ATP} currents by interaction with the Kir6.2 subunit.

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Abbreviations: HEK, human embryonic kidney; K_{ATP}, ATP-sensitive potassium channel; Kir6.2, inward rectifier potassium channel subunit 6.2; SUR1, sulphonylurea receptor type 1

Introduction

The electrical state of a cell can be coupled to its metabolic condition through modulation of ATP-sensitive potassium (K_{ATP}) channel activity. Consequently, these channels, which are present in many tissues, are considered to play an important role in both normal and diseased physiology including control of muscle function, neurological disorders and insulin secretion (Ashcroft & Ashcroft, 1990). Pharmacological manipulation of K_{ATP} channels is of obvious therapeutic value, clearly demonstrated in the treatment of hypo- and hyperglycaemia (Ashford, 1990). Sulphonylureas such as tolbutamide and glibenclamide are routinely used to treat non-insulin dependent diabetes mellitus (NIDDM) by stimulating insulin secretion, whereas the inhibition of insulin release by K_{ATP} openers such as diazoxide is sometimes used in the treatment of persistent hyperinsulinaemic hypoglycaemia of infancy (Dunne *et al*., 1997).

The molecular architecture of the pancreatic β cell K_{ATP} channel complex has been proposed to consist of at least two distinct proteins, a pore forming inwardly rectifying potassium channel sub-unit (Kir6.2) and an associated modulatory sulphonylurea receptor (SUR1). Current models propose that the K_{ATP} channel is an octomeric complex with four Kir6.2 sub-units, which constitute the channel pore, and four SUR1 sub-units which endow the channel with sensitivity to the sulphonylureas (Tucker & Ashcroft, 1998; Babenko *et al*., 1998). Heterologous expression of Kir6.2 and SUR1 in *Xenopus* oocytes, have demonstrated that expression of Kir6.2 or SUR1 alone does not give rise to functional K_{ATP} channels but when Kir6.2 and SUR1 are co-expressed, large inwardly rectifying currents are observed in response to application of metabolic inhibitors such as sodium azide (Gribble *et al*., 1997a).

The pharmacology of azide-induced currents appears to mimic that of native pancreatic β cell K_{ATP} channels as Kir6.2-SUR1 currents are inhibited by the sulphonylureas tolbutamide and glibenclamide and activated by diazoxide (Gribble *et al*., 1997a). Furthermore truncation of Kir6.2 by up to 36 carboxy-terminal amino acids induces channel expression in the absence of SUR1 and this has shown that the site of interaction for ATP is present on Kir6.2 alone, and that sensitivity to sulphonylureas, diazoxide and the potentiation of channel activity by Mg-ATP requires co-expression of the truncated Kir6.2 with SUR1 (Tucker *et al*., 1997). However, not all drugs require the presence of SUR1 to enable inhibition of the pore-forming subunit, Kir6.2, as the imidazolines (Proks & Ashcroft, 1997) and the antiarrhythmic agent, cibenzoline (Mukai *et al*., 1998) block Kir6.2 currents in the absence of SUR1.

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Two other, structurally unrelated, agents have also recently been shown to inhibit β-cell-type K_{ATP} channel activity. Thiazolidinediones, used to enhance peripheral insulin sensitivity, such as troglitazone (Lee *et al*., 1996a) and englitazone (Rowe *et al*., 1997) and the putative anti-depressant and anorectic agent, ciclazindol (Lee *et al*., 1996b), block K_{ATP} channels in the insulin secreting cell line, CRI-G1. The inhibition of K_{ATP} channel currents by both englitazone and ciclazindol is unaffected by removal of intracellular Mg^{2+} ions and after trypsinization of the cytoplasmic surface of excised patches, treatments known to abolish sulphonylurea sensitivity (Lee *et al*., 1994a,b), indicating that these drugs may act at a site distinct from the sulphonylureas.

It is generally considered that sodium azide activates K_{ATP} currents by depleting intracellular ATP through uncoupling mitochondrial oxidative phosphorylation by inhibition of cytochrome oxidase (Tsubaki & Yoshikawa, 1993). However, recently it has been shown that sodium azide activates K_{ATP} channels in the absence of any metabolic-induced decline of ATP levels, indicating a more direct effect on this channel type (Harvey *et al*., 1999). Consequently, using whole-cell and single channel recordings of Kir6.2-SUR1 currents from *Xenopus* oocytes and HEK 293 cells we have examined whether azide directly activates Kir6.2-SUR1 channels and determined the sensitivity of the cloned K_{ATP} channel to the sulphonylureas, tolbutamide and glibenclamide in comparison to englitazone and ciclazindol, in the presence and absence of azide. In addition we have addressed the question as to which subunit is responsible for the actions of these agents.

Methods

Preparation of in vitro *cRNA and cDNAs for transfection*

cDNAs encoding mouse Kir6.2 and a C-terminal truncated form of Kir6.2, missing the last 26 amino acids, Kir6.2Δ26 (provided by Professor F. Ashcroft, University of Oxford), and rat SUR1 (provided by Dr G. Bell, University of Chicago) were used in this study. *In vitro* cRNA was prepared from Kir 6.2 and SUR1 cDNAs. One μg of linear template cDNA was used to prepare capped cRNA by *in vitro* transcription using the mMessage mMachine (Ambion Inc.) according to the manufacturer's instructions. The cRNAs were aliquoted and stored in DEPC-treated distilled water at $-70^{\circ}C$ until required. Endotoxin free Kir 6.2, SUR1 and Kir6.2Δ26 cDNAs for transfection was prepared using a Qiagen endotoxin free plasmid kit (Qiagen).

Oocyte collection and preparation

Ovarian lobes were removed from mature female *Xenopus laevis* frogs (Blades Biological) after killing the animal by destruction of the brain. Oocytes were separated by treatment with 1 U ml^{-1} collagenase (Type 1A, Sigma) for 60–90 min at room temperature. Defollicated stage V–VI oocytes were selected for injection using a stereomicroscope on the basis of size, morphology and clear differentiation of animal and vegetal poles. Oocytes were injected either with 50 nl of a 1 : 1 mix of Kir6.2 and SUR1 cRNAs ($\sim$1 ng of each cRNA) or 50 nl of water as a control. Isolated oocytes were maintained at 18°C in modified Barth's solution containing (mM): NaCl 110, KCl 1, $NaHCO_3$ 2.4, Tris-HCl 7.5, $Ca(NO_3)_2$ 0.41, $CaCl_2$ 0.41, $MgSO_4$ (pH 7.4) 0.82 supplemented with 50 mg ml^{-1} gentamycin (Sigma) and 550 mg ml^{-1} pyruvic acid (Sigma). Currents were studied 2–5 days post-injection.

Cell culture and transfection

HEK 293 cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies) containing 1000 mg l^{-1} glucose and 110 mg l^{-1} sodium pyruvate with pyridoxine 4 mg l^{-1} supplemented with 10% foetal bovine serum (Helena Biosciences) and 1% penicillin/streptomycin (Life Technologies). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and plated onto 35 mm culture dishes (Falcon 3001) such that after 24 h the dishes were 40% confluent prior to transfection. Each dish was co-transfected with either 1.4 μg of a 1 : 1 ratio of Kir6.2Δ26/CD4 cDNAs or 3.15 μg of a 1 : 1 ratio of Kir6.2Δ26/SUR1/CD4 cDNAs using Fugene 6 transfection reagent (Roche Diagnostics Ltd.). A ratio of 2 : 3 μg cDNA to μl Fugene 6 was used as per the manufacturer's protocol. The culture media was replaced after 4 h and the cells were used for recording 24–48 h post-transfection. Transfected cells were selected by visible binding of anti-CD4 coated beads (Dynal, Oslo) following incubation with the beads for 20 min.

Drugs and solutions

Sodium azide, adenosine-5′-triphosphate (magnesium salt), glibenclamide and tolbutamide were purchased from Sigma. Ciclazindol was obtained from Wyeth, englitazone sodium was a gift from Pfizer and troglitazone a gift from Parke-Davis. Sodium azide was prepared as a 1 M stock solution in water, stock solutions of tolbutamide (100 mM), glibenclamide (10 mM), ciclazindol (10 mM) and englitazone (100 mM) were prepared in DMSO.

Electrophysiological recording and analysis

Oocytes: Whole cell currents were measured using a two electrode voltage clamp technique. Voltages were applied and currents measured using a Macintosh computer running HEKA software connected to a GeneClamp 500 amplifier (Axon Instruments) *via* a Instutech ITC-16 interface. Both current and voltage electrodes were filled with 3 M KCl and had resistances of 0.5–2.0 MΩ. To minimize series resistance errors a virtual bath ground was used with a reference electrode in the bath and a second bath electrode, filled with 3 M KCl, positioned close to the oocyte. Recordings were made in a high-potassium bath solution, KD96 containing (mM): KCl 96, NaCl 2, $CaCl_2$ 1.8, HEPES 5 (pH 7.4 with KOH). Working concentrations of drugs were prepared in KD96 and superfused into the bath. Experiments were carried out at room temperature, 18–24°C. In order to construct concentration-response relations for drug-induced inhibition of whole-cell currents, the slope conductance was determined from the linear portion (-100 to -10 mV) of the current-voltage relation in control (with azide) and for a single concentration of drug. Concentration-response curves were fitted by non-linear regression to the following equation

$$I/I_c = 1/(1 + (a/b)^{n}{}_H) \quad (1)$$

where a = half maximal inhibitory concentration, b = drug concentration and n_H = Hill coefficient.

Single channel recordings were made from oocytes injected with Kir6.2 and SUR1 cRNA as above. Two to five days post-injection oocytes were placed in hypertonic stripping solution (which consisted of (mM): K^+ aspartate

200, KCl 20, $MgCl_2$ 1, EGTA 10, HEPES 10 (pH 7.4 with KOH) for 5 min and the vitelline membrane removed manually. Naked oocytes were transferred to modified Barth's solution prior to use. Single channel recordings were made from excised patches in the inside-out configuration using electrodes with 5–8 MΩ resistances. Currents were recorded with a GeneClamp 500 amplifier (Axon Instruments), filtered at 1 kHz and recorded onto digital audio tape. The pipette solution contained (mM): KCl 140, $MgCl_2$ 1.2, $CaCl_2$ 2.6 and HEPES 10 (pH 7.4 with KOH) and the bath solution contained (mM): KCl 110, $MgCl_2$ 1.44, KOH 30, EGTA 10 and HEPES 10 (pH 7.2 with KOH).

Single channel recordings were made from inside-out patches obtained from transiently transfected HEK 293 cells selected as previously described. Recording pipettes (8–12 MΩ) were filled with solution containing (mM) KCl 140, $CaCl_2$ 1, $MgCl_2$ 1, HEPES-KOH 10 (pH 7.2), and the bath solution contained (mM) KCl 140, $MgCl_2$ 1, $CaCl_2$ 2, EGTA 10, HEPES-KOH 10. Single channel currents were measured using an EPC-7 patch clamp amplifier (List Electronics), filtered at 1 kHz and recorded onto digital audio tape, and replayed for illustration on a Gould TA240 chart recorder.

ATP and drugs were diluted from stock solutions into the internal solutions as indicated in the text and added to the bath by superfusion. Single channel data were analysed for current amplitude (I) and average channel activity ($N_f.P_o$, where N_f is the number of functional channels in the patch and P_o, the open state probability) as described previously (Lee *et al*., 1995). All data are expressed as the mean ± s.e.mean and statistical analyses were performed using unpaired student's *t*-test.

Results

Oocytes injected with water, Kir6.2 or SUR1 alone or co-injected with Kir6.2 and SUR1 cRNAs exhibited no currents different to those of non-injected oocytes (Figure 1a). As previously reported (Gribble *et al*., 1997a) the application of sodium azide (3 mM) for 10–15 min induced significant current only from oocytes co-injected with both Kir6.2 and SUR1 (Figure 1b) and this was reversible on wash-out of the sodium azide (data not shown). The mean current amplitude at −100 mV was 0.027 ± 0.03 μA before, and −12.9 ± 0.9 μA ($n = 57$) 15 min after application of 3 mM azide. This increase in current has been attributed to the azide causing metabolic inhibition, resulting in decreased intracellular ATP content, and subsequent activation of an ATP-sensitive K^+ current (Gribble *et al*., 1997a). The whole-cell current induced by azide is almost completely blocked by application of 1 μM glibenclamide ($n = 3$; Figure 1b) or 0.5 mM tolbutamide ($n = 6$; Figure 1d), effects that are not readily reversible on washout of the drugs. Figure 1c shows the corresponding current (I)–voltage (V) relations for an oocyte injected with Kir6.2 + SUR1 cRNA in the absence and presence of 3 mM sodium azide and following addition of 1 μM glibenclamide. Inhibition of these whole-cell currents by the sulphonylureas support the notion that azide has induced the activation of K_{ATP} channel currents in oocytes co-injected with Kir6.2 and SUR1.

However, it has recently been demonstrated that activation of native K_{ATP} channels in the rat insulinoma cell line, CRI-G1, by sodium azide is not dependent upon a decline in cellular ATP content and that azide can activate K_{ATP} channel currents in isolated membrane patches (Harvey *et al*., 1999). Conse-

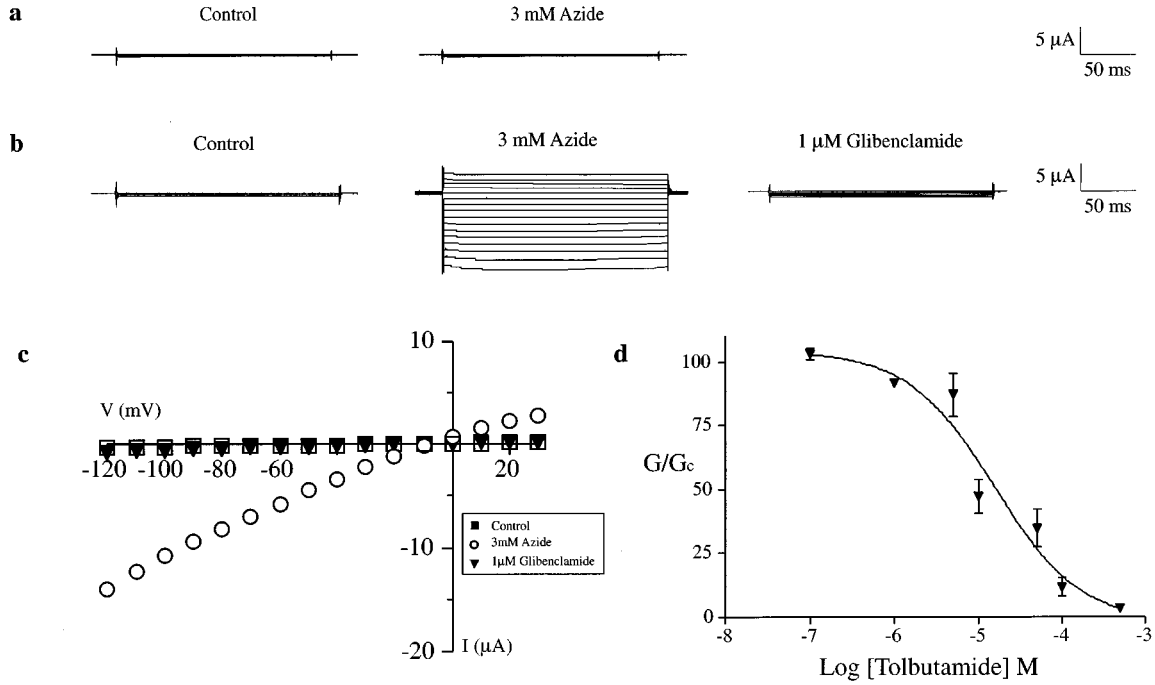

Figure 1 Activation of potassium current by sodium azide. (a,b) Whole cell currents recorded from two separate oocytes injected with (a) water and (b) cRNA encoding Kir6.2 and SUR1 before (control) and after exposure to 3 mM sodium azide for 15 min. Currents were elicited by a series of voltage steps from −120 mV to +30 mV from a holding potential of −10 mV, in this and subsequent figures. In (b) the azide-activated current is shown to be blocked following exposure to 1 μM glibenclamide in the presence of 3 mM sodium azide. (c) Corresponding current-voltage relations for the currents obtained in (b). (d) Concentration-inhibition curve for tolbutamide upon K_{ATP} channel currents activated by azide. Data are expressed as fractions of the control slope conductance (G_C). All points are the means of between four and six separate experiments and the vertical lines show the s.e.mean. The values for IC_{50} were obtained by fitting the data by non-linear regression.

quently we have examined the action of 3 mM sodium azide when applied directly to inside-out patches isolated from oocytes expressing Kir6.2-SUR1 currents. Inside-out patches excised from uninjected control oocytes exhibited no K_{ATP} channel activity ($n=4$). In contrast, patches excised from Kir6.2-SUR1 injected oocytes displayed significant channel activity, characterized, in symmetrical K^+ recording conditions, by an approximately linear current-voltage relationship at hyperpolarized potentials in symmetrical K^+ recording conditions with a mean single channel conductance of 69.1 ± 0.01 pS ($n=6$) and exhibiting mild inward rectification at depolarized voltages (Figure 2a). In control experiments on inside-out patches, channel activity was demonstrated to be inhibited by ATP in a reversible manner with 0.5 and 1 mM ATP causing a $70.0 \pm 4.2\%$ ($n=3$) and an $86.5 \pm 4.9\%$ ($n=3$) inhibition of activity respectively (Figure 2b). Tolbutamide (0.5 mM) also inhibited these currents, causing a $94.9 \pm 1.4\%$ ($n=4$) reduction in activity, an action reversible on washout of the tolbutamide in isolated patches. These data indicate that co-expression of Kir6.2 and SUR1 induces the appearance of single potassium channel currents with the properties particular of K_{ATP} channels. Application of 3 mM sodium azide, in the presence of 0.1 mM MgATP, to the cytoplasmic aspect of inside-out patches induced a rapid (between 30–120 s) increase ($492 \pm 222\%$) in K_{ATP} channel activity ($n=8$; Figure 2c). The corresponding mean values of channel activity in the presence of 0.1 mM MgATP and following application of sodium azide were 0.50 ± 0.24 and 1.94 ± 0.72 respectively ($n=8$; $P<0.05$). The azide-induced increase in channel activity was readily reversed on washout of the azide, maintaining the presence of 0.1 mM MgATP (0.73 ± 0.31; $n=8$). These data demonstrate that, in the presence of MgATP, sodium azide induces a rapid and reversible activation of Kir6.2-SUR1 currents in excised patches comparable to that reported for native K_{ATP} channels in CRI-G1 insulin-secreting cells (Harvey *et al*., 1999).

Initial examination of Kir6.2-SUR1 pharmacology was performed on whole cell Kir6.2-SUR1 currents induced by 3 mM sodium azide. In the presence of azide, tolbutamide (0.1 μM – 0.5 mM) was applied to voltage-clamped oocytes and engendered a concentration dependent, and irreversible, reduction in mean current (data not shown). From the tolbutamide concentration-inhibition curve, the IC_{50} value and Hill coefficient were 15.3 μM and 0.84 respectively (Figure 1d) which are similar values to that reported for tolbutamide inhibition of native pancreatic β-cell K_{ATP} channels (Ashcroft & Ashcroft, 1990) and for Kir6.2-SUR1 currents expressed in *Xenopus* oocytes (Gribble *et al*., 1997c, 1998).

In marked contrast, Kir6.2-SUR1 currents are relatively insensitive to inhibition by ciclazindol in comparison to native K_{ATP} channels (Figure 3). For example, in a separate series of experiments on oocytes injected with Kir6.2-SUR1, the mean resting current, at a membrane potential of -100 mV, was 0.29 ± 0.1 μA, which increased to 15.2 ± 2.3 μA in the presence

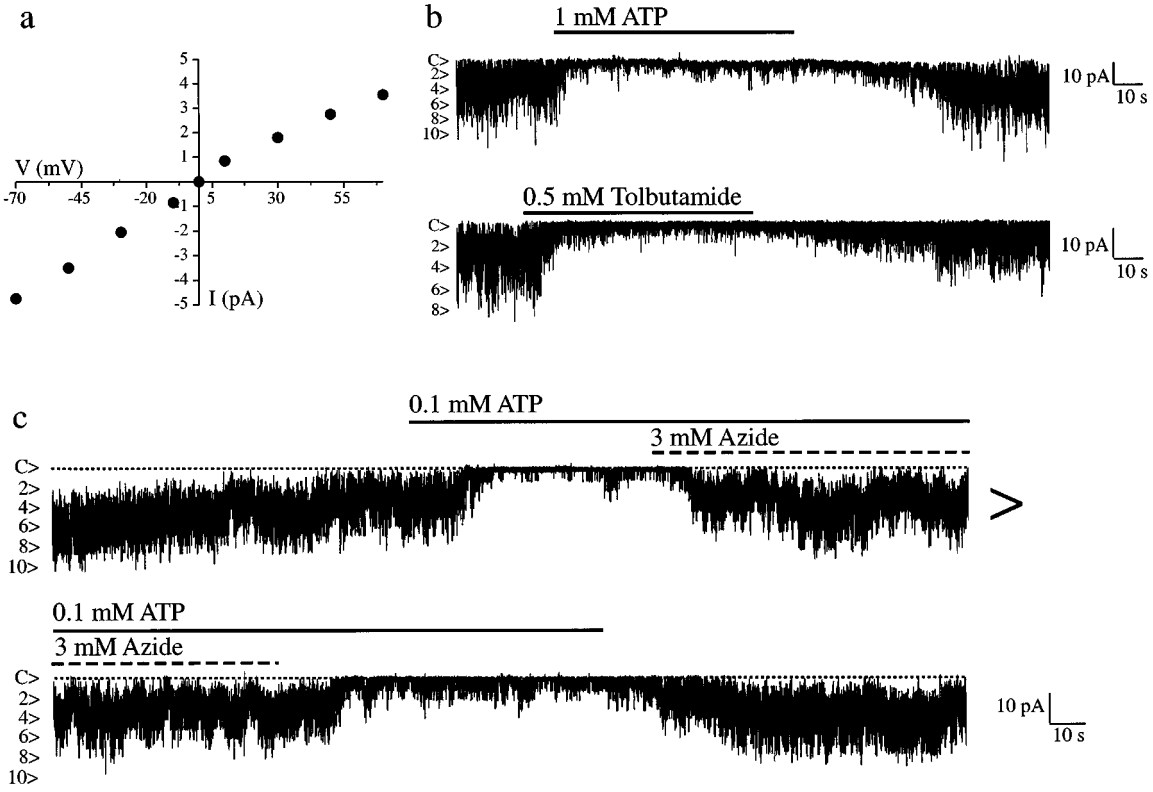

Figure 2 Sodium azide activates single K_{ATP} channels. (a) Current-voltage relationship for K_{ATP} channel currents recorded, under symmetrical KCl conditions, from an inside-out patch excised from a *Xenopus* oocyte co-injected with cRNA encoding Kir6.2 and SUR1. (b) Continuous single channel currents recorded from an inside-out patch at a holding potential of -50 mV exposed to symmetrical 140 mM KCl. Application of either 1 mM Mg-ATP (top current trace) or 0.5 mM tolbutamide (lower current trace, separate patch) inhibited channel activity. The N_fP_o values were 2.59 and 0.30 in control and after addition of 1 mM Mg-ATP, which recovered to 3.41 on washout. The values were 1.41, 0.04 and 1.32 for control, 0.5 mM tolbutamide and wash respectively. (c) Single channel currents recorded from an inside-out patch under the same conditions as (b). > indicates continuity of the trace. Application of 0.1 mM Mg-ATP inhibited channel activity with N_fP_o decreasing from 3.36 to 0.20. Addition of 3 mM sodium azide in the continued presence of 0.1 mM Mg-ATP reversibly activated channel currents; N_fP_o increased to 4.07 in the presence of azide and decreased to 0.64 on wash out of sodium azide in the continued presence of 0.1 mM Mg-ATP. On complete wash out of the Mg-ATP, N_fP_o increased to 3.96.

of 3 mM sodium azide. Application of 50 μM ciclazindol, a concentration sufficient to completely block native K_{ATP} channels in CRI-G1 insulin-secreting cells (Lee *et al*., 1996b), resulted in a mean whole cell current at -100 mV of 12.9 ± 2.5 μA ($n=8$) a level not significantly ($P>0.1$) different from control. Higher concentrations of ciclazindol (0.5 mM)

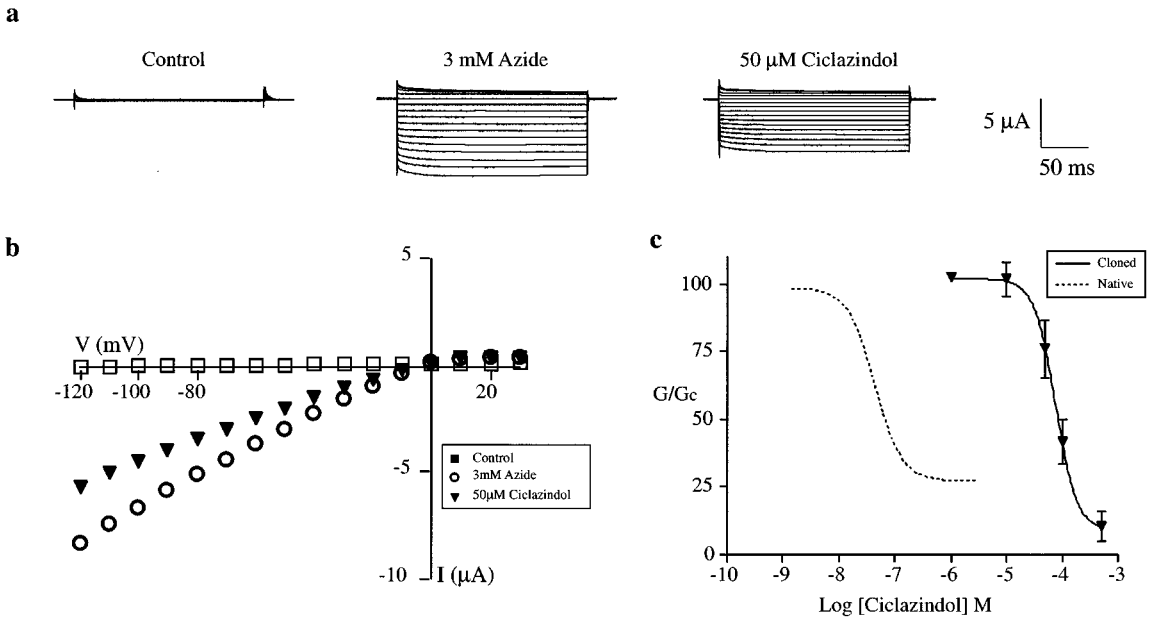

Figure 3 Effect of ciclazindol on whole cell currents. (a) Whole cell currents recorded from a *Xenopus* oocyte injected with cRNA encoding Kir6.2 and SUR1 under control conditions, after 15 min exposure to 3 mM sodium azide and 15 min later, following exposure to 50 μM ciclazindol in the presence of 3 mM sodium azide. (b) Corresponding current-voltage relations for the currents obtained in (a). (c) Concentration-inhibition curve for ciclazindol upon K_{ATP} channel currents activated by azide. Data are expressed as fractions of the control slope conductance (G_C). All points are the means of between three and seven separate experiments and the vertical lines show the s.e.mean. The values for IC_{50} were obtained by fitting the data by non-linear regression. The dashed line represents the concentration-inhibition curve for ciclazindol on native K_{ATP} channels (from Lee *et al*., 1996b).

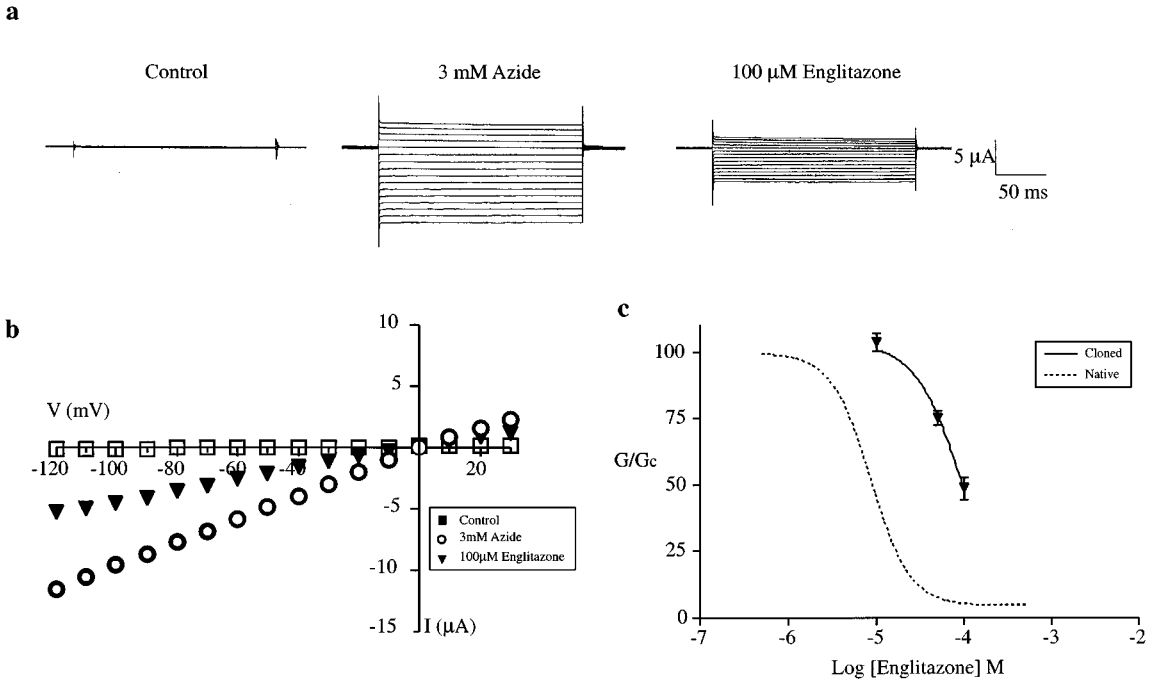

Figure 4 Effect of englitazone on whole cell currents. (a) Whole cell currents recorded from a *Xenopus* oocyte injected with cRNA encoding Kir6.2 and SUR1 under control conditions, after 15 min exposure to 3 mM sodium azide and 15 min later, following exposure to 100 μM englitazone in the presence of 3 mM sodium azide. (b) Corresponding current-voltage relations for the currents obtained in (a). (c) Partial concentration-inhibition curve for englitazone upon K_{ATP} channel currents activated by azide. Data are expressed as fractions of the control slope conductance (G_C). All points are the means of six separate experiments and the vertical lines show the s.e.mean. The values for IC_{50} were obtained by fitting the data by non-linear regression. The dashed line represents the concentration-inhibition curve for englitazone on native K_{ATP} channels (from Rowe *et al*., 1997).

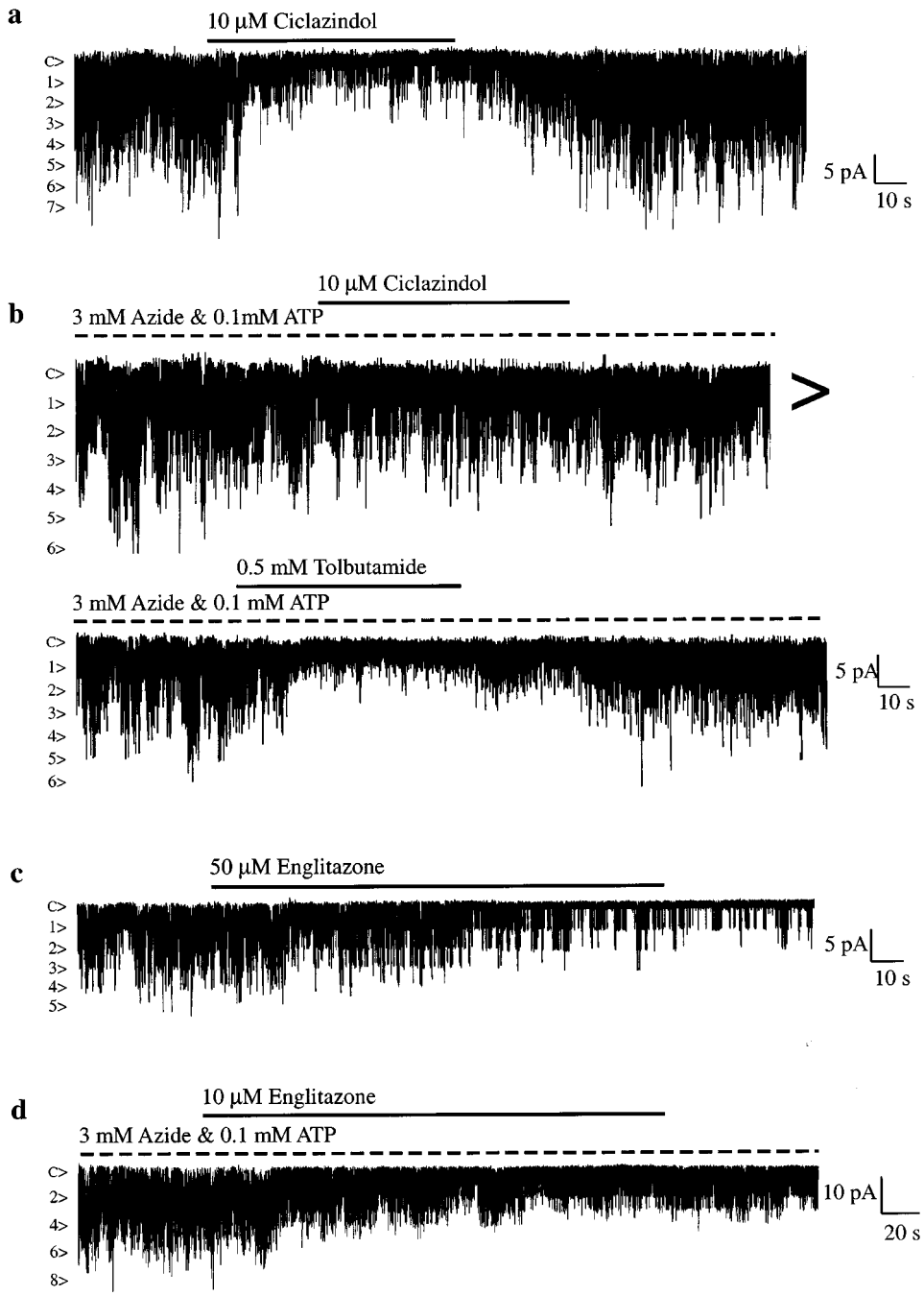

Figure 5 Sodium azide alters the sensitivity of single K_{ATP} channels to ciclazindol but not englitazone or tolbutamide. Single channel currents recorded from inside-out patches, excised from *Xenopus* oocytes co-injected with cRNA encoding Kir6.2 and SUR1, at a holding potential of −50 mV exposed to symmetrical 140 mM KCl. (a) In the absence of sodium azide, 10 μM ciclazindol (upper trace) inhibits cloned K_{ATP} channel activity. The N_fP_o values were 1.10, 0.11 and 0.90 for control, 10 μM ciclazindol and wash respectively. (b) Following activation by sodium azide in the presence of 0.1 mM Mg-ATP, 10 μM ciclazindol fails to inhibit cloned K_{ATP} channel currents. The N_fP_o values were 3.06, 2.71 and 2.24 for azide, 10 μM ciclazindol and wash respectively. In contrast the sensitivity to the sulphonylurea tolbutamide (lower trace) is unaffected by sodium azide activation. The N_fP_o values were 2.43, 0.41, and 2.55 for azide, 0.5 mM tolbutamide and wash respectively. (c) Englitazone inhibits cloned K_{ATP} channel activity in the absence of sodium azide. The N_fP_o values were 0.22 and 0.07 for control and on addition of 50 μM englitazone respectively, no wash out of the drug was observed. (d) Englitazone also inhibited cloned K_{ATP} channel activity following activation by sodium azide. The values of N_fP_o were 2.37 and 1.21 for azide and on addition of 10 μM englitazone respectively, again no wash out of the drug was observed.

did significantly reduce the whole cell current, and from the concentration-response curve for azide-induced currents, the IC_{50} value and Hill coefficient were 75.6 μM and 2.3 respectively (Figure 3c). This IC_{50} value differs markedly from that reported previously using the CRI-G1 insulin-secreting cell line, values of 40 nM (Lee *et al*., 1996b) and 127 nM (Harvey & Ashford, 1998) being obtained.

Similarly, the thiazolidinedione, englitazone at concentrations which reduce, significantly, native K_{ATP} channel currents had a much reduced inhibitory influence on Kir6.2-SUR1 currents activated by sodium azide in oocytes. For example, addition of 10 μM englitazone, a concentration close to that which half-maximally inhibits native K_{ATP} channel currents in insulin-secreting cells (Rowe *et al*., 1997; Harvey & Ashford,

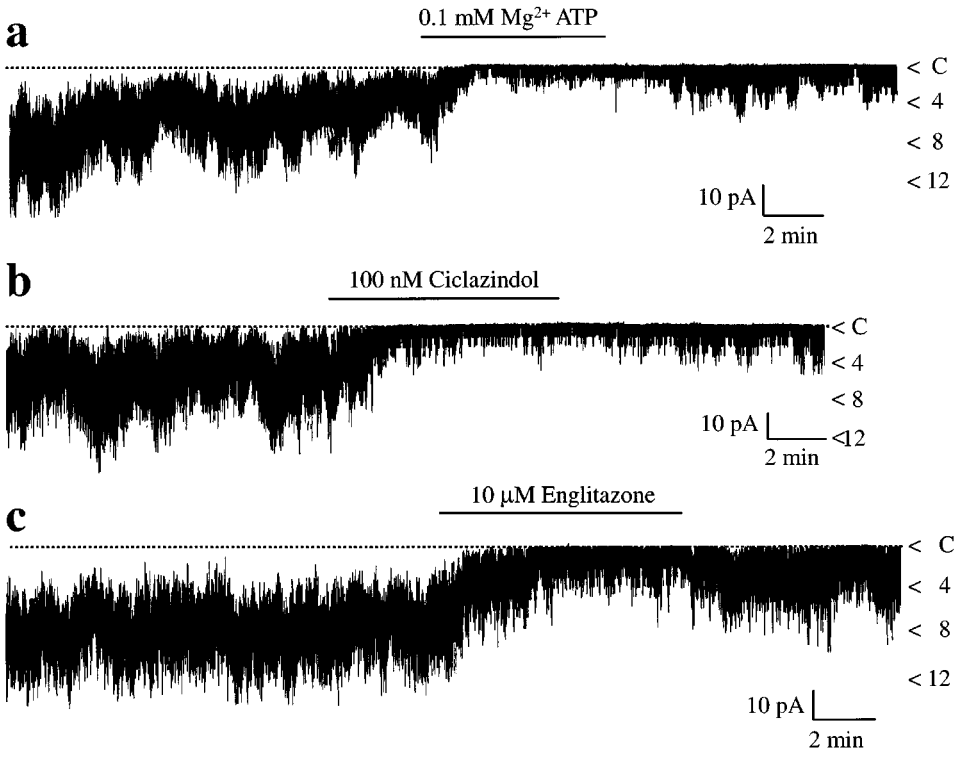

Figure 6 Single channel currents recorded from inside-out patches excised from HEK 293 cells transiently expressing Kir6.2Δ26+SUR1. Patches were held at a membrane potential of −40 mV in symmetrical 140 mM KCl. Application of (a) 0.1 mM Mg-ATP, (b) 100 nM ciclazindol and (c) 10 μM englitazone reduced channel activity, with poor recovery following washout of these agents. The values of N_fP_o were (a) 4.22 and 0.27 for control and 0.1 mM Mg-ATP respectively, (b) 4.69 and 0.37 for control and 100 nM ciclazindol respectively, and (c) 6.84 and 1.53 for control and 10 μM englitazone respectively.

1998), had no significant effect on azide-induced Kir6.2-SUR1 currents. However, application of higher concentrations of englitazone (50 and 100 μM) did inhibit Kir6.2-SUR1 whole cell currents (Figure 4a–c), an action not reversed on washout of drug, with an estimated IC_{50} value and Hill coefficient of 93.6 μM and 1.7 respectively. Thus, azide-activated macroscopic Kir6.2-SUR1 currents expressed in oocytes do not completely replicate the pharmacological sensitivity observed for native pancreatic β-cell type K_{ATP} channels.

It was possible that the presence of the sodium azide, used to activate Kir6.2-SUR1 currents in intact oocytes, was responsible for some or all of the difference in pharmacological sensitivity between the cloned channel currents and native K_{ATP} channels of pancreatic ß-cells. Consequently we examined the sensitivity of Kir6.2-SUR1 single channel currents in excised inside-out patches to inhibition by ciclazindol and englitazone in the absence and presence of sodium azide. Excised inside-out patch recordings were made in symmetrical (140 KCl in pipette and bath) K^+-containing solution at a membrane potential of −50 mV. In the absence of sodium azide, application of 10 μM ciclazindol to Kir6.2-SUR1 currents resulted in a substantial inhibition (87.7±3.2%) of channel activity, an action reversible on washout of the drug (Figure 5a). The mean level of channel activity obtained in control conditions was 1.09±0.19, which declined to 0.12±0.03 in the presence of 10 μM ciclazindol ($n=8$; $P<0.01$). In contrast, in inside-out patches exposed to 3 mM sodium azide and 0.1 mM MgATP, addition of 10 μM ciclazindol resulted in no significant change in mean channel activity (Figure 5b); the mean value prior to ciclazindol was 1.66±0.54 and in the presence of 10 μM ciclazindol was 1.55±0.58 ($n=12$; $P>0.8$). A higher concentration of ciclazindol (100 μM) also had no significant effect on sodium azide-activated Kir6.2-SUR1 current ($n=3$; data not shown). Clearly, activation of the current by sodium azide dramatically depressed the efficacy of ciclazindol at inhibiting Kir6.2-SUR1 current. However this is not the case for englitazone as application of this drug to inside-out membrane patches inhibits Kir6.2-SUR1 currents in the absence and presence of sodium azide with equal potency. For example, 10 and 50 μM englitazone caused a 67.6±10.8% ($n=3$) and 81.5±5.8% ($n=7$) inhibition of Kir6.2-SUR1 currents in the absence of azide (Figure 5c) whereas in the presence of 3 mM sodium azide and 0.1 mM MgATP (Figure 5d) the inhibition was 47.0±10.3% ($n=5$) and 88.8±3.6% ($n=5$) respectively. This inhibition was also not reversed following washout of the drug.

These data indicate that the presence of sodium azide, used to activate Kir6.2-SUR1 currents in oocytes, results in a marked reduction in the efficacy of Kir6.2-SUR1 current to inhibition by ciclazindol,. The discrepancy in efficacy for englitazone between macroscopic currents recorded from intact oocytes and that of isolated patches from oocytes or K_{ATP} currents recorded from native tissue may be related to the use of the intact oocyte and/or slow access of the drug to its site of action. It has also been argued that both ciclazindol and englitazone inhibit pancreatic β-cell type K_{ATP} channels by acting at a site distinct from the sulphonylureas (Lee *et al.*, 1996b; Rowe *et al.*, 1997). Consequently, we have examined the actions of these drugs on cloned K_{ATP} channel currents, expressed in human embryonic kidney (HEK 293) cells, using the truncated form of Kir6.2 (Kir6.2ΔC26; the last 26 amino acids deleted from the C-terminus), expressed alone (Tucker *et al.*, 1997) and co-expressed with SUR1, to determine which subunit confers drug sensitivity. Single channel currents were recorded, from inside-out membrane patches excised from HEK 293 cells transiently co-expressing Kir6.2ΔC26-SUR1 or

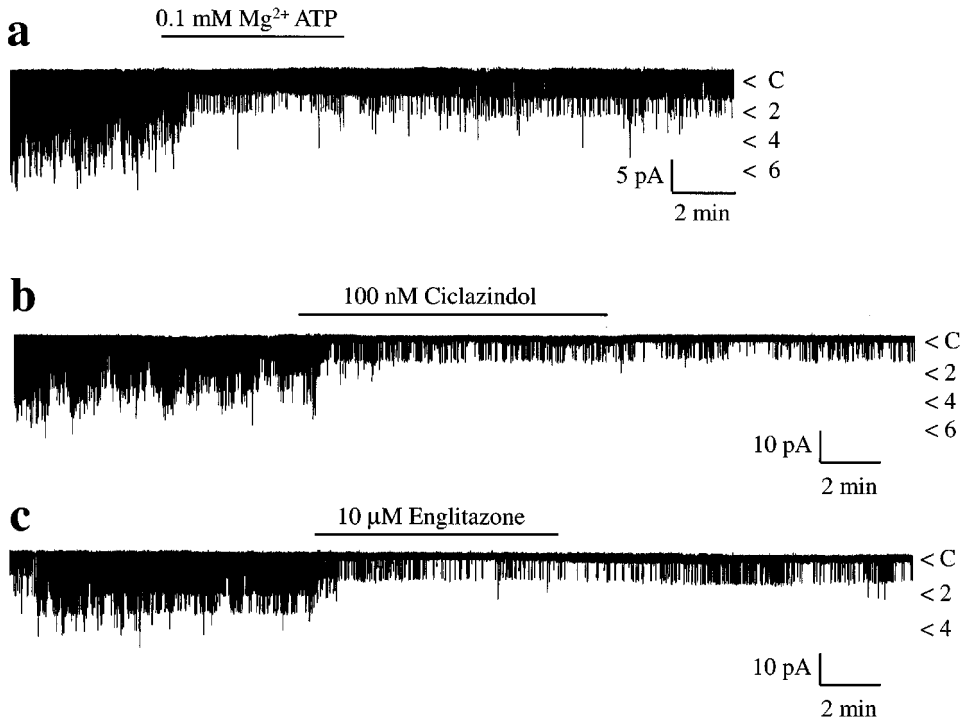

Figure 7 Single channel currents recorded from inside-out patches excised from HEK 293 cells transiently expressing Kir6.2Δ26 alone. Patches were held at a membrane potential of −40 mV in symmetrical 140 mM KCl. Application of (a) 0.1 mM Mg-ATP, (b) 100 nM ciclazindol and (c) 10 μM englitazone reduced channel activity, with poor recovery following washout of these agents. The values of N_fP_o were (a) 0.96 and 0.04 for control and 0.1 mM Mg-ATP respectively, (b) 0.45 and 0.004 for control and 100 nM ciclazindol respectively, and (c) 0.34 and 0.01 for control and 10 μM englitazone respectively.

Kir6.2ΔC26 alone, with the sensitivity of the currents to Mg-ATP being routinely examined to establish channel identity (Figures 6a and 7a). Application of 100 nM ciclazindol resulted in a significant ($P<0.05$) inhibition of channel activity when applied to patches expressing either Kir6.2ΔC26-SUR1 (Figure 6b) or Kir6.2ΔC26 currents (Figure 7b), inducing inhibition of channel activity by $79.0\pm3.1\%$ ($n=6$) and $84.9\pm6.3\%$ ($n=4$) respectively. Similarly, 10 μM englitazone induced significant ($P<0.05$) inhibition of both Kir6.2ΔC26-SUR1 (Figure 6c) and Kir6.2ΔC26 currents (Figure 7c); by $79.8\pm1.5\%$ ($n=5$) and $93.8\pm2.5\%$ ($n=3$) respectively. The effects of both these agents were not easily reversed following washout of the drugs. Therefore, ciclazindol and englitazone appear to be able to inhibit K_{ATP} currents expressed in HEK 293 cells independently of the presence of SUR1. Sodium azide (3 mM), in the presence of 0.1 mM Mg-ATP, did not increase channel currents composed of full length Kir6.2-SUR1 ($n=3$) or Kir6.2ΔC26-SUR1 ($n=3$) in inside-out patches in HEK 293 cells (data not shown).

Discussion

The characteristics of the currents induced by co-expression of cRNAs encoding Kir6.2 and SUR1 in *Xenopus* oocytes are in agreement with the previously published reports (Gribble *et al*., 1997a,b). The sensitivity of the expressed channel to agents that act to inhibit channel activity is similar to that of the native K_{ATP} channels of pancreatic β cells with respect to MgATP and the sulphonylureas glibenclamide and tolbutamide. However, the actions of sodium azide and the resultant alterations in the apparent pharmacological specificity and sensitivity of Kir6.2-SUR1 macroscopic currents raises some doubts as to the suitability of this method of evaluating drug potency. Clearly, the inhibitory actions of ciclazindol and englitazone, two structurally unrelated inhibitors of native pancreatic β-cell type K_{ATP} channels, are substantially altered when examined by this method.

Current convention propounds that cloned K_{ATP} whole-cell currents in oocytes are inhibited at resting intracellular ATP concentrations and can only be observed following metabolic inhibition of the oocyte (Gribble *et al*., 1997a). The metabolic inhibitor sodium azide, which has been routinely used to activate cloned K_{ATP} whole-cell currents, is proposed to inhibit mitochondrial electron transport resulting in a lowering of intracellular ATP levels. We demonstrate, however, that sodium azide not only can activate whole cell Kir6.2-SUR1 currents but can also, in the presence of MgATP, induce a rapid, reversible activation of Kir6.2-SUR1 currents in excised patches. This latter effect is not expected following the metabolic inhibition argument. Furthermore, levels of total ATP concentration in oocytes are not dramatically reduced by metabolic inhibition, even for periods up to 60 min (Gribble *et al*., 1997a). In a recent study it was also demonstrated that, in the presence of MgATP, sodium azide activates K_{ATP} channels in excised patches from CRI-G1 insulin-secreting cells (Harvey *et al*., 1999). Interestingly, this effect may not be isolated to sodium azide as 2,4 dinitrophenol (DNP) has been demonstrated to activate cardiac K_{ATP} channels independently of metabolic inhibition (Alekseev *et al*., 1997). Thus azide, like DNP, may activate K_{ATP} channels by an alternative mechanism unrelated to intracellular ATP depletion. Surprisingly, however, sodium azide had no activating action on isolated patches containing Kir6.2-SUR1 currents (or indeed on the truncated Kir6.2ΔC26 currents) when transiently expressed in HEK cells, suggesting that co-expression of

Kir6.2 and SUR1 in HEK 293 may not fully reconstitute the native environment for these channels. One explanation for these results is that another protein or factor is required in order to observe the sodium azide activation and this factor is present in the CRI-G1 insulin-secreting cells and *Xenopus* oocytes, but not the HEK cells used in the present study, or that it is lost/inactivated in the inside-out configuration.

The sensitivity of both pancreatic β-cell type K_{ATP} channel currents and heterologously expressed Kir6.2-SUR1 currents to tolbutamide was unaltered following azide activation. This is in contrast to the action of DNP which is reported to occlude sulphonylurea inhibition of K_{ATP} channel currents (Findlay, 1993; Guillemare *et al*., 1995; Alekseev *et al*., 1997). In contrast, the macroscopic Kir6.2-SUR1 currents, recorded from oocytes, are relatively insensitive to block by both ciclazindol and englitazone, compounds which inhibit, significantly, native K_{ATP} channels at the concentrations used in this series of experiments. It is clear from the inside-out patch experiments that the presence of azide markedly affects the sensitivity of the cloned channels to ciclazindol and it is likely that the same effect accounts for the reduced efficacy of this drug on the whole cell currents. The same is not true of englitazone where, although the whole cell currents show reduced sensitivity to this compound, inside-out patches exhibit sensitivity close to that of native K_{ATP} channels. It is possible that this apparent conflict is due to poor access of the drug in the intact oocyte.

Interestingly, the *ob* gene product, leptin, which activates K_{ATP} channels in both hypothalamic glucose-responsive neurones (Spanswick *et al*., 1997) and CRI-G1 cells (Harvey *et al*., 1997) has also been reported to cause a substantial reduction in the sensitivity of CRI-G1 K_{ATP} channel currents to these inhibitors (Harvey & Ashford, 1998). This latter study, which compared the effects of leptin and diazoxide, demonstrated that ciclazindol sensitivity was dramatically reduced following leptin application such that the EC_{50} values were 127 nM and 40 μM for diazoxide activated and leptin activated K_{ATP} channels respectively. Leptin also altered the sensitivity to englitazone, with EC_{50} values of 7.7 μM and 52 μM for diazoxide- and leptin-activated K_{ATP} channels respectively. Although we have previously alluded to the sodium azide-induced K_{ATP} channel activation having a great deal in common with that of diazoxide activation (Harvey *et al*., 1999), the results obtained above, particularly with respect to inhibition of K_{ATP} channel currents by ciclazindol, indicate that there may also be a common process in the mechanism by which azide and leptin activate K_{ATP} channels.

Sulphonylureas have been clearly shown to act *via* the high affinity sulphonylurea receptor, SUR1, but the sites of action by which ciclazindol and englitazone mediate their effects on K_{ATP} channels are unclear, although there is evidence that these sites are distinct from that of the sulphonylureas. For example previous studies indicate that treatments which cause functional uncoupling of K_{ATP} channel currents from inhibition by the sulphonylureas do not alter the channel sensitivity to inhibition by ciclazindol or englitazone (Lee *et al*., 1996b, Rowe *et al*., 1997). In addition ciclazindol does not displace ^{3}H-glibencamide binding from CRI-G1 (Lee *et al*., 1996b) or porcine brain (Noack *et al*., 1992) membranes. The present results using HEK 293 cells transfected with a truncated form of Kir6.2, Kir6.2ΔC26, demonstrate unequivocally that the site of action of both ciclazindol and englitazone is distinct from the sulphonylurea receptor and most likely lies on the Kir6.2 subunit. Furthermore, the presence of SUR1 appears to have no action on the potency of these drugs to inhibit currents generated by Kir6.2. This mirrors the results obtained for cibenzoline inhibition of cloned K_{ATP} channel currents where it was demonstrated to be equally effective at inhibiting Kir6.2ΔC26 and Kir6.2ΔC26 + SUR1 currents (Mukai *et al*., 1998). However, the observation that sodium azide-activated Kir6.2-SUR1 currents expressed in *Xenopus* oocytes are differentially sensitive to ciclazindol, but not englitazone, in the isolated patch configuration indicates that these inhibitors are unlikely to be acting at the same site on Kir6.2.

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