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Diazoxide- and leptin-activated K_{ATP} currents exhibit differential sensitivity to englitazone and ciclazindol in the rat CRI-G1 insulin-secreting cell line

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1 The effects of the antidiabetic agent englitazone and the anorectic drug ciclazindol on ATP-sensitive K^+ (K_{ATP}) channels activated by diazoxide and leptin were examined in the CRI-G1 insulin-secreting cell line using whole cell and single channel recording techniques.

2 In whole cell current clamp mode, the hyperglycaemic agent diazoxide (200 μM) and the ob gene product leptin (10 nM) hyperpolarised CRI-G1 cells by activation of K_{ATP} currents. K_{ATP} currents activated by either agent were inhibited by tolbutamide, with an IC_{50} for leptin-activated currents of 9.0 μM.

3 Application of englitazone produced a concentration-dependent inhibition of K_{ATP} currents activated by diazoxide (200 μM) with an IC_{50} value of 7.7 μM and a Hill coefficient of 0.87. In inside-out patches englitazone (30 μM) also inhibited K_{ATP} channel currents activated by diazoxide by $90.8 \pm 4.1\%$.

4 In contrast, englitazone (1–30 μM) failed to inhibit K_{ATP} channels activated by leptin, although higher concentrations (>30 μM) did inhibit leptin actions. The englitazone concentration inhibition curve in the presence of leptin resulted in an IC_{50} value and Hill coefficient of 52 μM and 3.2, respectively. Similarly, in inside-out patches englitazone (30 μM) failed to inhibit the activity of K_{ATP} channels in the presence of leptin.

5 Ciclazindol also inhibited K_{ATP} currents activated by diazoxide (200 μM) in a concentration-dependent manner, with an IC_{50} and Hill coefficient of 127 nM and 0.33, respectively. Furthermore, application of ciclazindol (1 μM) to the intracellular surface of inside-out patches inhibited K_{ATP} channel currents activated by diazoxide (200 μM) by $86.6 \pm 8.1\%$.

6 However, ciclazindol was much less effective at inhibiting K_{ATP} currents activated by leptin (10 nM). Ciclazindol (0.1–10 μM) had no effect on K_{ATP} currents activated by leptin, whereas higher concentrations (>10 μM) did cause inhibition with an IC_{50} value of 40 μM and an associated Hill coefficient of 2.7. Similarly, ciclazindol (1 μM) had no significant effect on K_{ATP} channel activity following leptin addition in excised inside-out patches.

7 In conclusion, K_{ATP} currents activated by diazoxide and leptin show different sensitivity to englitazone and ciclazindol. This may be due to differences in the mechanism of activation of K_{ATP} channels by diazoxide and leptin.

Keywords: K_{ATP} channels; diazoxide; leptin; englitazone; ciclazindol

Introduction

It is well established that ATP-sensitive potassium (K_{ATP}) channels play a central role in the control of insulin secretion from pancreatic beta cells (Ashcroft & Rorsman, 1991). Closure of these channels results in membrane depolarization, activation of voltage-dependent calcium channels and ultimately the exocytotic release of insulin (Ashford, 1990). Thus drugs that modulate K_{ATP} channel function are important therapeutic tools in the treatment of hyperglycaemia and hypoglycaemia.

Recently the thiazolidinedione-derived agent englitazone was developed for the treatment of non-insulin dependent diabetes mellitus (NIDDM). Englitazone and other thiazolidinediones are thought to enhance the action of insulin without stimulating insulin secretion (Fujita *et al*., 1988; Stevenson *et al*., 1990; Sohda *et al*., 1992). However, seemingly contrary to this, recent studies show that englitazone inhibits K_{ATP} channels in CRI-G1 insulinoma cells, an action consistent with stimulation of insulin secretion(Rowe *et al*., 1997). In addition troglitazone, another thiazolidinedione derivative has been shown to inhibit K_{ATP} channels in CRI-G1 insulin secreting cells (Lee *et al*., 1996a) and ventromedial hypothalamic neurons (Lee & Boden, 1997). The inhibitory action of englitazone appears to occur at a site distinct from that utilised by the antidiabetic sulphonylureas (Masuda *et al*., 1995; Rowe *et al*., 1997). The anorectic drug ciclazindol which was originally used as an antidepressant agent (Ghose *et al*., 1978) is a novel inhibitor of K_{ATP} channels in CRI-G1 insulin secreting cells (Lee *et al*., 1996b). Like englitazone, the inhibitory action of ciclazindol is thought to be mediated *via* a mechanism distinct from the sulphonylureas (Lee *et al*., 1996b).

In pancreatic beta cells (Dunne *et al*., 1989) and insulin secreting cell lines (Sturgess *et al*., 1988; Kozlowski *et al*., 1989), the benzothiadiazine diazoxide hyperpolarizes the cell membrane *via* activation of K_{ATP} channels. Although the mechanism underlying this action of diazoxide is not entirely clear, it is likely to act directly on the sulphonylurea receptor (SUR 1; Inagaki *et al*., 1996; Tucker *et al*., 1997). Recent reports indicate that the ob gene product leptin activates K_{ATP} channels in CRI-G1 insulinoma cells (Harvey *et al*., 1997a) and pancreatic beta cells (Keiffer *et al*., 1997), consistent with inhibition of insulin secretion. Leptin is a 167 amino acid protein that is released primarily from adipocytes and is

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thought to be an important satiety signal that regulates food intake and body weight (Zhang *et al*., 1994). The leptin receptor is a member of the class I cytokine receptor superfamily (Tartaglia *et al*., 1995) that usually signal *via* activation of tyrosine kinases. However recent studies in our laboratory suggest that leptin activation of K_{ATP} channels involves inhibition of tyrosine kinases and subsequent dephosphorylation of, as yet, unidentified proteins (Harvey & Ashford, 1998).

In the present study, we have examined the sensitivity of englitazone and ciclazindol on K_{ATP} channels activated by diazoxide and leptin. We present data showing that englitazone and ciclazindol are more potent inhibitors of K_{ATP} channels activated by diazoxide, as opposed to leptin. Some of these data have been published previously (Harvey & Ashford, 1997b).

Methods

Cell culture

Cells from the insulin-secreting cell line CRI-G1 were grown in Dulbecco's modified medium with sodium pyruvate and glucose, supplemented with 10% fetal calf serum and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were passaged every 2–5 days as described previously (Carrington *et al*., 1986).

Electrophysiological recording and analysis

Experiments were performed using whole cell current and voltage clamp recording configurations to monitor membrane potential and macroscopic currents, respectively and excised inside-out recordings to monitor single channel activity as described previously (Harvey *et al*., 1997a). Whole cell experiments were maintained in current clamp mode to monitor cell resting membrane potential with short excursions into voltage clamp mode to examine macroscopic current-voltage relationships. In voltage clamp recordings, cells were voltage clamped at −50 mV and 10 mV voltage steps of 100 ms duration were applied every 200 ms (range −120 mV to −30 mV). Current and voltage were measured using the Axon 200B amplifier and the currents evoked in response to the voltage step protocol were analysed using pClamp 6.0 software (Axon Instruments, Foster City, California). Single channel data were analysed for current amplitude (I) and average channel activity (Nf.Po) as described previously (Lee *et al*., 1995). Current clamp and single channel data were recorded onto digital audio tapes and replayed for illustration on a Gould TA 240 chart recorder.

Recording electrodes were pulled from borosilicate glass capillaries and had resistances of 1–5 MΩ for whole cell recordings and 8–12 MΩ for single channel experiments when filled with electrolyte solution. The pipette solution for whole cell recordings comprised (mM): 140 KCl, 0.6 $MgCl_2$, 2.73 $CaCl_2$, 5.0 ATP, 10.0 EGTA, 10 HEPES, pH 7.2 (free Ca^{2+} of 100 nM), whereas for single channel experiments it contained (mM): 140 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.2. The bath solution for whole cell recordings comprised of normal saline (mM); 135 NaCl, 5 KCl, $MgCl_2$, 1 $CaCl_2$, 10 HEPES, pH 7.4, whereas for inside-out excised patches the bath solution contained (mM): 140 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 EGTA, 10 HEPES, pH 7.2 (free Ca^{2+} of 30 nM). The free Ca^{2+} concentrations were calculated using the 'METLIG' programme (P. England & R. Denton, University of Bristol, U.K.). All solution changes were achieved by superfusing the bath with a gravity feed system at a rate of 10 ml/min which allowed complete bath exchange within 2 min. All experiments were performed at room temperature (22–25°C).

In the whole cell recording mode, drug effects were quantified by measuring the amplitude of current responses (I) during drug exposure and comparing them with those observed under control conditions (Ic). Values for the controls were obtained by calculating the mean amplitude before and after drug application. The concentration inhibition curves were fitted by non-linear regression to the following equation:

$$I/I_c = 1/(1 + (a/b)^{n_H})$$

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

Drugs

Englitazone and ciclazindol were gifts from Pfizer and Wyeth, respectively. Diazoxide, ATP and tolbutamide were obtained from Sigma. Recombinant human leptin was supplied by Dr Peter Lind of Pharmacia-Upjohn (Stockholm, Sweden). Leptin was prepared as a stock solution in normal saline and further diluted in normal saline containing 0.2% bovine serum albumin as a carrier. Tolbutamide was made up as a 100 mM stock solution in DMSO, and diazoxide as a stock solution in 0.1 mM KOH. ATP was made up as a 100 mM stock solution in 10 mM HEPES at pH 7.2 and kept at −4°C until required. Ciclazindol and englitazone sodium were prepared as 10 mM stock solutions in DMSO.

Statistical analysis

All data are expressed as the mean ± s.e.mean and statistical analyses were performed using unpaired student's *t*-test (unless otherwise stated).

Results

Diazoxide and leptin activate K_{ATP} channels

Under current clamp conditions with 5 mM ATP in the electrode, the mean resting membrane potential of CRI-G1 insulinoma cells was −38 ± 0.8 mV ($n = 59$). Application of the hyperglycaemic agent, diazoxide (200 μM; Trube *et al*., 1986) hyperpolarized CRI-G1 cells to −76 ± 0.8 mV ($n = 29$; Figure 1a). Examination of the voltage-clamped macroscopic currents indicate that the diazoxide-induced hyperpolarization is accompanied by an increase in the slope conductance from a control value of 0.58 ± 0.04 nS to 10.5 ± 0.96 nS following exposure to diazoxide ($n = 29$; $P < 0.05$; Figure 1a). The mean reversal potential associated with this increase in conductance was −79 ± 0.4 mV ($n = 8$) which is close to the calculated value for E_K of −84 mV under these conditions. The sulphonylurea tolbutamide (100 μM) completely reversed the membrane hyperpolarization and increase in conductance to pre-diazoxide levels ($n = 8$), indicating that these actions of diazoxide are attributable to activation of K_{ATP} channels. These data are in agreement with previous studies using this cell line (Sturgess *et al*., 1988; Kozlowski *et al*., 1989).

Similarly under current clamp conditions following dialysis with 5 mM ATP, application of ob gene product leptin (10 nM) resulted in hyperpolarization of CRI-G1 cells to −77 ± 1.1 mV with a concomitant increase in slope conductance from 0.56 ± 0.03 nS to 6.19 ± 0.81 nS ($n = 23$; $P < 0.05$; Figure 1b).

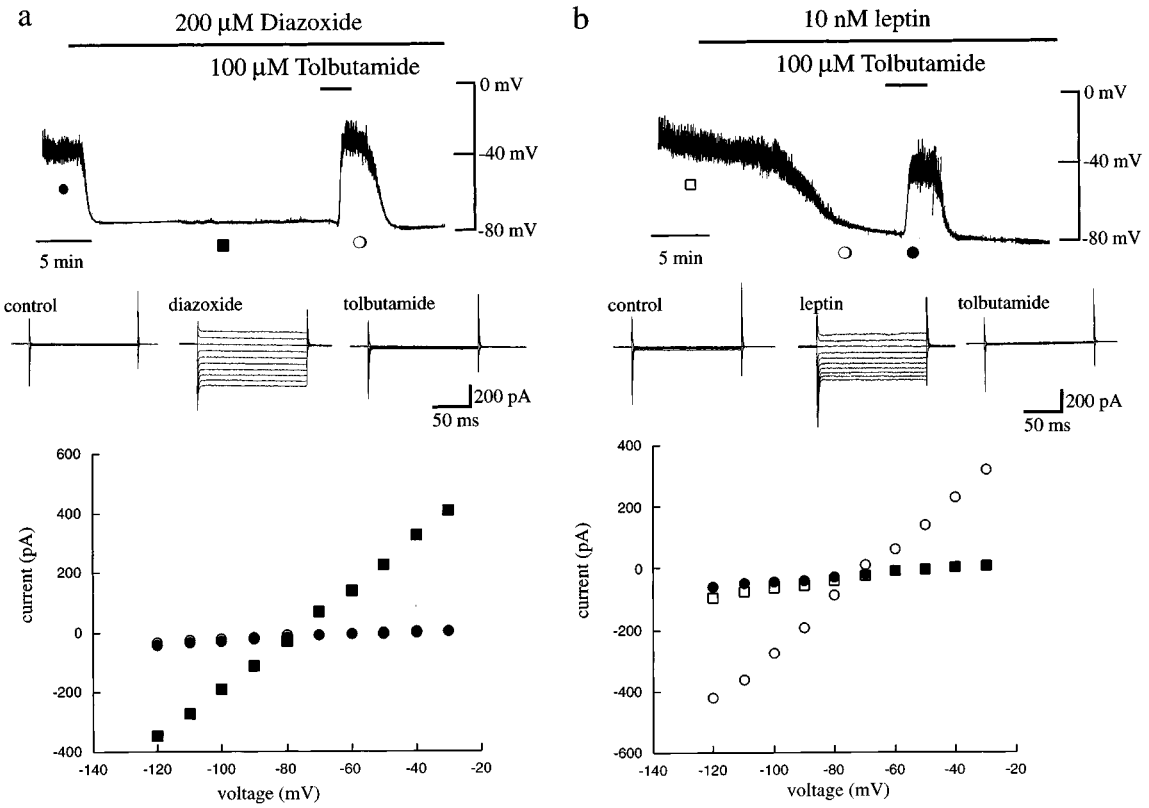

Figure 1 Diazoxide and leptin hyperpolarize CRI-G1 cells via activation of K_{ATP} channel currents. (a) The upper panel is a whole cell current clamp record of a CRI-G1 cell dialyzed with an electrode solution containing 5 mM ATP. In this and subsequent figures, the trace begins approximately 5 min after obtaining the whole cell configuration. Application of diazoxide (200 μM) for the time indicated resulted in rapid hyperpolarization of the membrane from -40 mV to -77 mV, an action readily reversed by the sulphonylurea tolbutamide (100 μM). The lower panel shows the individual voltage clamped current traces and a plot of the current-voltage relations for the currents obtained at the points specified in (a): control (●); diazoxide (■) and diazoxide and tolbutamide (○). Diazoxide increased the membrane conductance relative to control and tolbutamide (100 μM) reversed the diazoxide-induced increase in conductance with a reversal potential of -79 mV. (b) The top panel is a current clamp record of a cell dialyzed with 5 mM ATP. Addition of leptin (10 nM) hyperpolarized CRI-G1 cells from -41 mV to -80 mV, an action readily reversed by sulphonylurea tolbutamide (100 μM). The lower panel shows the individual voltage-clamped current traces and the current-voltage plot for the currents obtained in (b): control (□), leptin (○) and leptin and tolbutamide (●). Leptin increased the membrane conductance relative to control and tolbutamide reversed this action of leptin with a reversal potential of -78 mV.

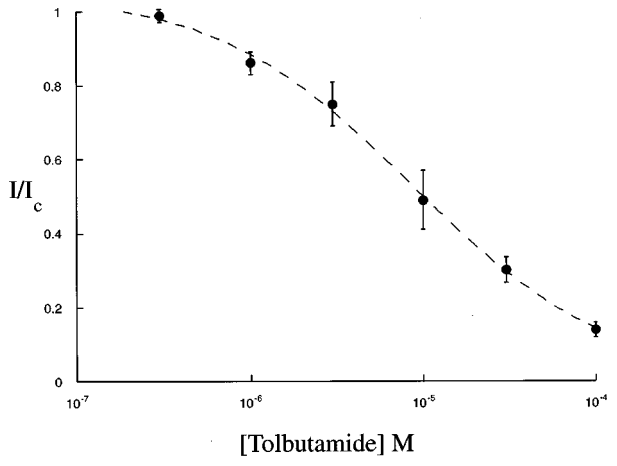

Figure 2 Concentration inhibition curve for tolbutamide upon K_{ATP} channel currents activated by leptin. Data are expressed as fractions of control currents (Ic) and this was calculated by comparing the total current evoked in response to the voltage step protocol (as described in Methods) following leptin activation of K_{ATP} channels and in the combined presence of leptin and tolbutamide. All points are the means of between four and five separate experiments and the vertical lines show the s.e.mean. The values for IC_{50} (half maximal inhibitory concentration) were obtained by fitting the data by non-linear regression.

This was due to opening of K_{ATP} channels as the sulphonylurea tolbutamide (0.3–100 μM) reversed the leptin-induced hyperpolarization and increase in conductance in a concentration-dependent manner (Figure 2). At a concentration of 10 μM, tolbutamide depolarized CRI-G1 cells by 16 ± 3.1 mV ($n=4$) and reduced the slope conductance from 6.3 ± 1.3 nS to 3.5 ± 0.78 nS ($n=4$; $P<0.05$), with a reversal potential of -79 ± 2.3 mV ($n=4$). From the tolbutamide concentration inhibition curve, the IC_{50} value and Hill coefficient were 9.0 μM and 0.80, respectively (Figure 2), which are similar values to those reported previously (Sturgess *et al.*, 1988; Lee *et al.*, 1994a). Together these data indicate that leptin activates K_{ATP} channels in agreement with previous studies using this cell line (Harvey *et al.*, 1997a).

Effects of englitazone on membrane potential and whole cell currents

Englitazone (0.3–100 μM) caused a concentration dependent and reversible inhibition of K_{ATP} channels activated by diazoxide ($n=20$; Figure 3a). Englitazone at a concentration of 10 μM resulted in approximately 50% reduction in the diazoxide-induced hyperpolarization and increase in K^+ conductance. Thus englitazone (10 μM) reversed the membrane hyperpolarization (-75 ± 3.6 mV; $n=5$) and increase

in slope conductance (from 0.59 ± 0.05 nS to 6.3 ± 0.69 nS; $n=5$; $P<0.05$) induced by diazoxide to -56 ± 2.5 mV and 2.9 ± 0.45 nS, respectively ($n=5$). The reversal potential associated with this action of englitazone was -78 ± 1.8 mV ($n=5$), consistent with a reduction in K^+ conductance. From the concentration inhibition curve, englitazone was found to have a half maximal inhibitory concentration (IC_{50}) of 7.7 μM and an associated Hill coefficient of 0.87 (Figure 3c). These values correlate well with those reported for the inhibitory action of englitazone of K_{ATP} channels activated following

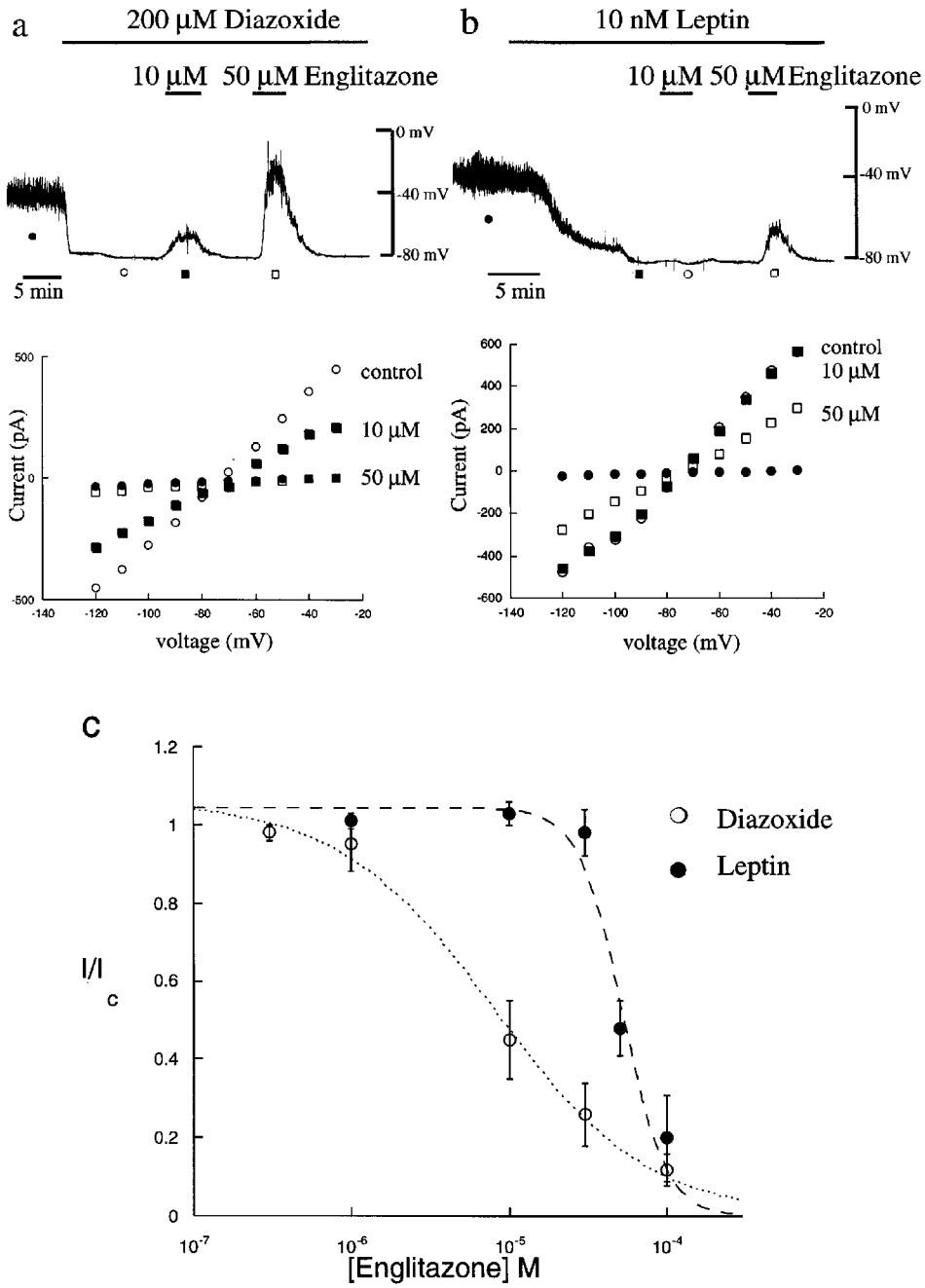

Figure 3 Effects of englitazone on membrane potential and whole cell currents. (a) The upper panel is a current clamp record of a cell dialyzed with a 5 mM ATP-containing electrode solution. Application of diazoxide (200 μM) resulted in hyperpolarization of CRI-G1 cells from -41 mV to -80 mV. Englitazone (10 μM) applied after diazoxide caused around 50% reduction in the diazoxide-induced hyperpolarization, whereas englitazone (50 μM) completely reversed the actions of diazoxide. The lower panel is a plot of the current-voltage relations for the voltage clamped currents obtained at the time points specified in the upper panel: Control (●); diazoxide (○); englitazone (10 μM; ■) and englitazone (50 μM; □). Diazoxide increased the membrane conductance relative to control. Addition of englitazone after exposure to diazoxide resulted in around 50% (10 μM) and 100% (50 μM) inhibition of the diazoxide increase in conductance. (b) The upper panel is a current clamp record of a CRI-G1 cell dialyzed with 5 mM ATP. Application of leptin (10 nM) hyperpolarized the cell membrane from -39 mV to -79 mV. Subsequent addition of englitazone (10 μM) failed to effect the membrane potential. However englitazone (50 μM) caused about 50% reduction in the leptin-induced hyperpolarization. The lower panel is a plot of the current-voltage relations for the voltage clamped currents in (b): control (●); leptin (■); englitazone (10 μM; ○) and englitazone (50 μM; □). (c) Concentration inhibition curves for englitazone on K_{ATP} channels activated by either diazoxide (○) or leptin (●). Data are expressed as the fraction of control current (Ic) and all points on the curves correspond to the mean $\pm$ s.e.mean, values obtained from four or five individual experiments. The IC_{50} values were obtained by non-linear regression.

dialysis with a solution with no added ATP (Rowe *et al.*, 1997; IC_{50} of 8 μM).

In contrast to the actions of diazoxide, englitazone (1–100 μM) was less potent at inhibiting the leptin-induced membrane hyperpolarization and increase in slope conductance. Thus at low concentrations englitazone (1–30 μM) failed to inhibit K_{ATP} channels activated following the addition of leptin ($n=15$; Figure 3b). The membrane potential (-75 ± 30 mV) and slope conductance (5.9 ± 1.1 nS) values obtained following the addition of leptin (10 nM) were not significantly different from those obtained in the presence of 10 μM englitazone (-76 ± 3.1 mV and 6.0 ± 0.9 nS; $P>0.05$ paired *t*-test; $n=5$). However higher concentrations of englitazone (>30 μM) did inhibit the actions of leptin in a reversible manner ($n=8$). Following the leptin-induced hyperpolarization (to -74 ± 2.8 mV) and increase in slope conductance (to 5.8 ± 1.4 nS) application of englitazone (50 μM) depolarized the CRI-G1 cell membrane by 13 ± 1.6 mV with a concomitant reduction in slope conductance to 2.7 ± 0.9 nS ($n=4$). From the englitazone concentration inhibition curve the calculated IC_{50} value and Hill coefficient were 52 μM and 3.2, respectively (Figure 3c).

Effects of englitazone on single K^+ channels

In order to confirm that the englitazone-induced reduction in K^+ current observed in the whole cell configuration was due to blockade of K_{ATP} channels, the effects of englitazone on excised inside-out patches were examined. In this series of experiments cells were incubated with either diazoxide or leptin for 20–25 min prior to formation of the inside-out configuration to enable maximal activation of K_{ATP} channels. Recordings were made in symmetrical (140 KCl in pipette and bath) K^+-containing solution and the membrane potential was held at +40 mV. Following incubation with diazoxide (200 μM) addition of englitazone (30 μM) to the intracellular surface of excised inside-out patches produced a marked inhibition of channel activity that was partially reversed on washout ($n=4$; Figure 4a). Analysis of the total channel current (over a 120 s period) 1–3 min after formation of the inside-out configuration (control) and 2–4 min after the addition of englitazone (30 μM) resulted in mean channel activity (Nf.Po) of 0.41 ± 0.08 and 0.023 ± 0.007, respectively ($n=4$; $P<0.05$). The mean single channel current amplitude (I) did not change throughout the duration of the experiment. The potency of englitazone at inhibiting K_{ATP} channels activated by diazoxide in this study correlates well with the reported potency of englitazone at inhibiting K_{ATP} channels in excised inside-out patches in this cell line (Rowe *et al.*, 1997).

In contrast to these findings, englitazone (30 μM) was less effective at inhibiting K_{ATP} channels activated by prior incubation with the ob gene product leptin (10 nM; $n=3$; Figure 4b). Analysis of the total channel current (over 120 s) at the same time points as before indicated that the average K_{ATP} channel activity (Nf.Po) in the absence and presence of englitazone (30 μM) was $0.37\pm0.13\%$ and $0.37\pm0.16\%$, respectively ($n=3$: $P>0.05$).

Effects of ciclazindol on membrane potential and whole cell currents

Ciclazindol (1 nM–10 μM) inhibited K_{ATP} channels activated by diazoxide in a concentration-dependent and reversible manner ($n=28$; Figure 5). At a concentration <1 μM ciclazindol partially reversed the action of diazoxide. However, ciclazindol (10 μM) completely reversed the membrane

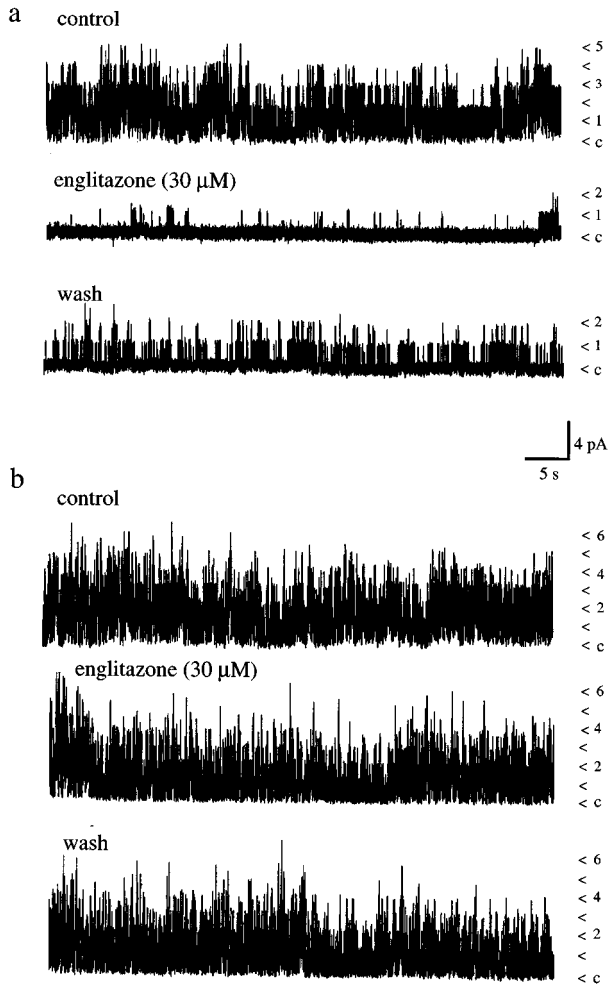

Figure 4 Effects of englitazone on single channel currents. Single channel currents recorded from inside-out membrane patches exposed to symmetrical 140 mM KCl at a membrane potential of +40 mV. Single channel openings are denoted by upward deflections. (a) Englitazone (30 μM) inhibited K_{ATP} channels activated by prior exposure to diazoxide (200 μM). This action of englitazone was partially reversed on washout. The Nf.Po values obtained in control conditions, in the presence of englitazone and following washout were 0.45, 0.042 and 0.13, respectively. (b) Englitazone (30 μM) had very little effect on K_{ATP} channels activated by the ob gene product leptin (10 nM). The Nf.Po values were as follows: control 0.68; englitazone 0.63; wash 0.60.

hyperpolarization induced by diazoxide (200 μM; $n=5$) from -76 ± 2.1 mV to -39 ± 2.8 mV ($P<0.05$). Similarly ciclazindol (10 μM) reduced the diazoxide-induced increase in slope conductance from 8.6 ± 0.81 nS to 0.71 ± 0.08 nS ($n=5$; $P<0.05$). The reversal potential associated with this action of ciclazindol was -79 ± 1.7 mV ($n=5$; Figure 5b), indicating the involvement of a K^+ conductance. From the concentration inhibition curve ciclazindol had an IC_{50} value of 127 nM and a Hill coefficient of 0.33 (Figure 5c). These data indicate that ciclazindol is less potent at inhibiting K_{ATP} channels activated by diazoxide rather than activation of K_{ATP} channels following dialysis with an electrode solution with no added ATP (Lee *et al.*, 1996b; IC_{50} value of 40 nM).

In contrast to its sensitivity for diazoxide, ciclazindol was much less potent at inhibiting K_{ATP} activated by leptin (10 nM; Figure 6). Low concentrations of ciclazindol (10 nM–3 μM) failed to reverse the membrane hyperpolarization and increase in conductance induced by leptin ($n=13$; Figure 6a). Thus the

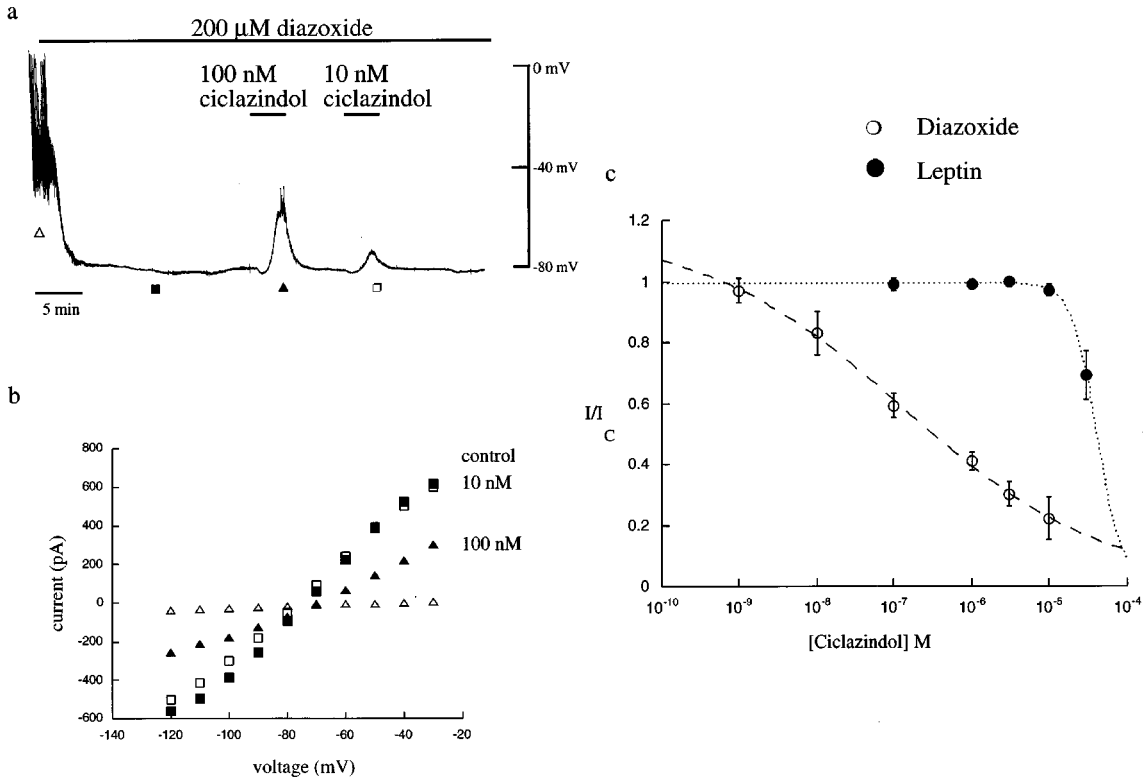

Figure 5 Effects of ciclazindol on the diazoxide-induced hyperpolarization and increased K^+ conductance. (a) is a current clamp record of a CRI-G1 cell dialyzed with 5 mM ATP. Application of diazoxide (200 μM) for the time indicated resulted in hyperpolarization of the cell membrane from -38 mV to -81 mV. Subsequent addition of ciclazindol at 100 nM and 10 nM resulted in reductions of around 50% and 25% in the diazoxide-induced hyperpolarization, respectively. (b) is a plot of the current-voltage relations for the points indicated in (a): control (△), diazoxide (■), diazoxide and ciclazindol (10 nM; □) and diazoxide and ciclazindol (100 nM; ▲). Ciclazindol (100 nM) reduced the diazoxide increase in membrane conductance by about 50% whereas ciclazindol (10 nM) caused about 25% reduction in K^+ conductance induced by diazoxide. (c) is the concentration inhibition curves for ciclazindol on K_{ATP} channel currents activated by either diazoxide (○) or leptin (●). Data are expressed as the fraction of control current (Ic) and all points on the curves correspond to the mean$\pm$s.e.mean values obtained from four or five individual experiments. The IC_{50} values were obtained by non-linear regression.

membrane potential (-76 ± 2.6 mV) and slope conductance (6.7 ± 1.2 nS) values obtained following application of leptin did not differ significantly from those obtained (-75 ± 2.9 mV and 6.5 ± 1.5 nS) in the presence of 3 μM ciclazindol ($n=4$; $P>0.05$; paired t-test). However, higher concentrations of ciclazindol ($\geqslant10$ μM) did inhibit the actions of leptin in a reversible manner ($n=9$). In the presence of 10 nM leptin, the cell membrane potential values obtained in the absence and presence of 30 μM ciclazindol were -73 ± 3.2 mV and -65 ± 2.9 mV, respectively ($n=4$; $P<0.05$). This action of ciclazindol (30 μM) was associated with a reduction in the slope conductance from 7.1 ± 1.8 nS to 4.6 ± 1.2 nS ($n=4$; $P<0.05$). The reversal potential associated with this action was -80 ± 1.3 mV ($n=4$). The IC_{50} value and Hill coefficient calculated from the ciclazindol concentration inhibition curve were 40 μM and 2.7, respectively (Figure 5c).

Effects of ciclazindol on single K^+ channels

Following incubation with diazoxide (200 μM) and subsequent formation of the inside-out configuration, addition of ciclazindol (1 μM) to the intracellular surface caused a marked reduction in K_{ATP} channel activity ($n=4$; Figure 7a). The mean channel activity obtained in the absence and presence of ciclazindol (1 μM) at the same time points as before was 0.67 ± 0.1 and 0.07 ± 0.004, respectively ($n=4$; $P<0.05$). In contrast, ciclazindol was much less potent at inhibiting K_{ATP} channels activated by leptin (10 nM; Figure 7b). The average K_{ATP} channel activity in the absence and presence of ciclazindol (1 μM) was 0.81 ± 0.06 and 0.77 ± 0.02, respectively ($n=3$, $P>0.05$).

Discussion

The present study shows that the hyperglycaemic agent diazoxide and the ob gene product leptin both cause hyperpolarization of CRI-G1 insulin-secreting cells, with an associated increase in K^+ conductance. This was due to activation of K_{ATP} channels as the sulphonylurea tolbutamide, at concentrations reported to maximally inhibit K_{ATP} channels in this cell line (Sturgess *et al.*, 1988; Lee *et al.*, 1994b), completely reversed the effects of both these agents. The half maximal inhibitory concentration observed in this study for tolbutamide inhibition of K_{ATP} channel currents activated by leptin corresponds well with its reported potency in the presence and absence of ATP (Trube *et al.*, 1986; Zunkler *et al.*, 1988). In contrast, the novel K_{ATP} channel inhibitor englitazone showed differential sensitivity in inhibiting K_{ATP} channels activated by diazoxide and leptin. The potency of englitazone (IC_{50} of 7.7 μM) at inhibiting the actions of diazoxide corresponds well to its potency at inhibiting K_{ATP} channels activated following dialysis with an electrode solution with no added ATP (Rowe *et al.*, 1997; IC_{50} of 8 μM). In

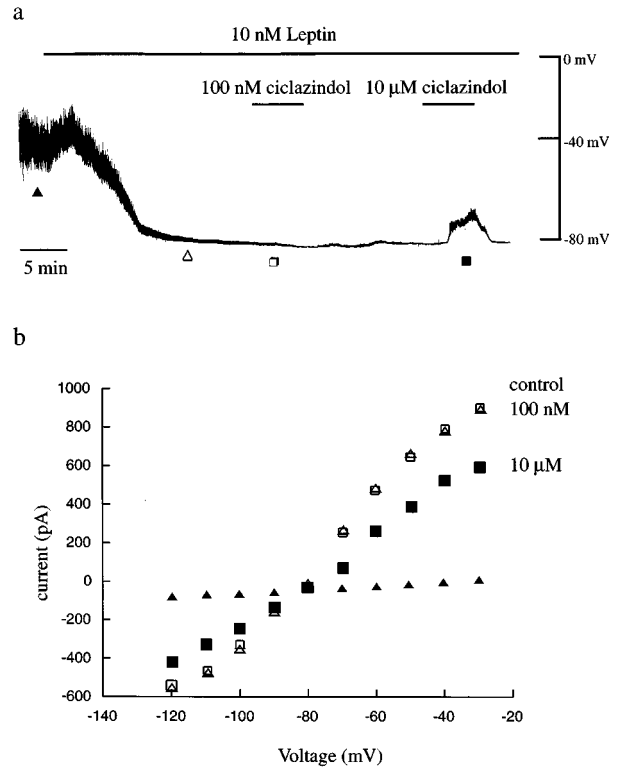

Figure 6 Effects of ciclazindol on leptin actions. (a) Is a current clamp record of a single cell dialyzed with an electrode solution containing 5 mM ATP. Addition of leptin (10 nM) hyperpolarized the cell membrane from −38 mV to −79 mV. Subsequent application of ciclazindol (100 nM) failed to reverse the leptin-induced hyperpolarization. Ciclazindol (10 μM) partially inhibited this action of leptin in a reversible manner. (b) is the current-voltage plot for the voltage-clamped macroscopic currents obtained in (a): control (▲), leptin (△), ciclazindol (100 nM; □), ciclazindol (10 μM; ■).

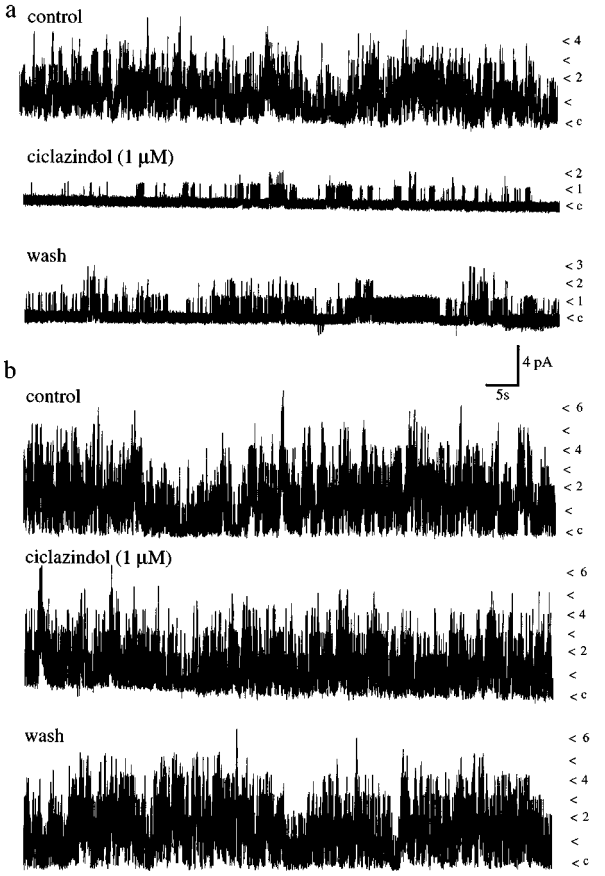

Figure 7 Effects of ciclazindol on single channel currents. Single channel currents recorded from inside-out membrane patches exposed to symmetrical 140 mM KCl and held at a membrane potential of +40 mV. Single channel openings are denoted by upward deflections (outward currents). (a) Ciclazindol (1 μM) inhibited the activity of K_{ATP} channels activated by diazoxide (200 μM), in a partially reversible manner. The values of Nf.Po were as follows: control 0.46; ciclazindol 0.033; wash 0.12. (b) Ciclazindol (1 μM) was much less effective at inhibiting K_{ATP} channels activated by leptin (10 nM). The values of Nf.Po were as follows: control 0.91; ciclazindol 0.87; wash 0.85. Note that there is little run down of K_{ATP} channels following activation of leptin.

contrast, however, englitazone was approximately 10 fold less potent at inhibiting K_{ATP} channels activated by leptin (IC_{50} of 52 μM). High concentrations of englitazone (>30 μM) were capable of inhibiting the actions of leptin, however this may be due to non-specific inhibition as this concentration range also causes inhibition of Ca^{2+} currents in this cell line (Rowe *et al.*, 1997). Similarly, the anorectic drug ciclazindol exhibits differential sensitivity in its inhibitory action on diazoxide (IC_{50} of 127 nM) and leptin (IC_{50} of 40 μM). Thus like englitazone, ciclazindol is a more potent inhibitor of K_{ATP} channels activated by diazoxide as opposed to leptin. Together these data suggest that diazoxide and leptin activate K_{ATP} channels *via* distinct mechanisms in CRI-G1 cells.

Previous studies have shown that englitazone (Rowe *et al.*, 1997) and ciclazindol (Lee *et al.*, 1996b) are novel inhibitors of K_{ATP} channels in the CRI-G1 insulin-secreting cell line. These agents appear to act at a site distinct from the sulphonylureas as functional uncoupling of SUR from K_{ATP} channels, either in the absence of Mg^{2+} ions (Lee *et al.*, 1994a) or by the addition of trypsin (Lee *et al.*, 1994b), does not alter the ability of either to inhibit K_{ATP} channels (Rowe *et al.*, 1997; Lee *et al.*, 1996b). Furthermore, ciclazindol at concentrations exceeding those required to maximally inhibit K_{ATP} channels, does not displace [^{3}H]-glibenclamide binding in CRI-G1 membrane fragments (Lee *et al.*, 1996b) or in porcine brain (Noack *et al.*, 1992). Furthermore the thiazolidinedione derivative, troglitazone has been shown to interact with SUR in a non-competitive manner, but only at higher concentrations than those required to stimulate insulin release (Masuda *et al.*, 1995). In view of these data, it is unlikely that englitazone, at the concentrations examined in this study, directly interacts with the sulphonylurea recognition site on SUR. The present data also support the notion that englitazone and ciclazindol act at a site distinct from the sulphonylureas. Both agents exhibit a much reduced potency at inhibiting K_{ATP} channel currents activated by leptin in contrast to the potency of tolbutamide (IC_{50} of 9 μM) which parallels previous reports of its potency for inhibition of K_{ATP} channels in this cell line (IC_{50} of 12 μM; Lee *et al.*, 1994b). However, examination of the actions of these inhibitors on heterologously expressed Kir 6.2/SUR 1 and on the 36 amino acid C-terminal deletion of Kir 6.2 (Tucker *et al.*, 1997) is clearly required.

The potency of englitazone at inhibiting K_{ATP} channels following dialysis with zero ATP (IC_{50} of 8 μM; Rowe *et al.*, 1997), parallels its potency at inhibiting diazoxide actions (IC_{50} of 7.7 μM) observed in this study. In contrast, however, ciclazindol was approximately 3 fold less potent at inhibiting K_{ATP} channels activated by diazoxide (IC_{50} value of 127 nM), compared to previous reports of its potency at K_{ATP} channels

(IC_{50} of 40 nM; Lee *et al*., 1996b). These data suggest that the mechanism of action of englitazone and ciclazindol may differ also. This contention is supported by the finding that englitazone, over the concentration range that inhibits K_{ATP} channels, also inhibits calcium-activated non-selective cation (NS_{Ca}) channels, in a voltage-independent manner (Rowe *et al*., 1997). In contrast, ciclazindol inhibits NS_{Ca} channels in a voltage-dependent manner but only at much higher concentrations than required to inhibit K_{ATP} channels (Lee *et al*., 1996b). Further experiments are required, however, to determine the precise sites of action of englitazone and ciclazindol.

In normal rats and NIDDM rodent models, englitazone has been shown to enhance the actions of insulin without stimulating insulin secretion from pancreatic beta cells (Fujuta *et al*., 1988; Stevenson *et al*., 1990). This is perhaps surprising since in this cell line englitazone inhibits K_{ATP} channels activated by either the hyperglycaemic agent diazoxide or following dialysis with zero ATP (Rowe *et al*., 1997). Previous studies have demonstrated that the anorectic drug ciclazindol inhibits K_{ATP} channels with a similar potency to second generation sulphonylureas such as glibenclamide (Lee *et al*., 1996b). However in insulin release studies, ciclazindol was not as effective at stimulating insulin secretion as the sulphonylureas (Lee *et al*., 1996b). These apparent anomalies may possibly be explained by the reduced potency of these agents observed in the presence of leptin, since leptin can activate K_{ATP} channels in this cell line (Harvey *et al*., 1997a) over a similar concentration range to that circulating in the body (Caro *et al*., 1996). This finding may be an important consideration in the use of englitazone in the treatment of NIDDM as hyperleptinaemia is commonly associated with this disease.

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