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Effect of englitazone on K_{ATP} and calcium-activated non-selective cation channels in CRI-G1 insulin-secreting cells

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1 The effects of englitazone sodium, an antidiabetic agent, on ion channel activity in the CRI-G1 insulin secreting cell line was examined by use of the patch clamp technique.

2 Application of englitazone to the outside of CRI-G1 cells in the whole-cell recording configuration produced concentration-dependent inhibition of K_{ATP} currents with an IC_{50} value of 8 μM. The inhibition of the K^+ current was not affected by the removal of Mg^{2+} ions from or the addition of trypsin to the solution bathing the intracellular surface of the cell membrane.

3 Englitazone also inhibited K_{ATP} channel activity in recordings from inside out excised membrane patches. The concentration-dependence of inhibition was identical to that observed in whole-cell recordings and was voltage-independent. Single channel recordings confirmed that neither the absence or presence of Mg^{2+} ions nor the addition of trypsin at the intracellular surface of the membrane influenced the inhibition of K_{ATP} channels by englitazone.

4 Englitazone also inhibited Ca^{2+}-activated non-selective cation (NS_{Ca}) channels in inside-out patches in a concentration-dependent and voltage-independent manner with an IC_{50} value of 10 μM. In comparison, the non-sulphonylurea K_{ATP} channel blocker ciclazindol produced a slight voltage-dependent inhibition of the NS_{Ca} channel at a concentration of 20 μM.

5 In whole-cell recordings englitazone, at a relatively high concentration (50 μM) in comparison with that required to block K_{ATP} and NS_{Ca} channels, inhibited voltage-activated Ca^{2+} currents by 33% but did not inhibit voltage-activated K^+ and Na^+ currents.

6 It is concluded that englitazone is a novel blocker of NS_{Ca} and K_{ATP} channels. The inhibition of K_{ATP} channels occurs following procedures that dissociate sulphonylurea receptor coupling to the channel. The equipotent and voltage-independent inhibition of NS_{Ca} and K_{ATP} channels by englitazone may indicate a common mechanism of block.

Keywords: Englitazone; K_{ATP} channel; non-selective cation channel; thiazolidinedione; CRI-G1

Introduction

Englitazone is one of a number of novel thiazolidinedione-derived orally active anti-diabetic agents being developed for the treatment of non-insulin dependent (type 2) diabetes mellitus (NIDDM). Patients with NIDDM and animal models of NIDDM are usually characterized by peripheral insulin resistance, aberrant pancreatice insulin secretion and enhanced hepatic glucose output (DeFronzo, 1988). It has been suggested that englitazone and other thiazolidinedione-derived anti-diabetic agents, such as troglitazone, act by enhancing the action of insulin without stimulating insulin secretion from the pancreas (Fujita *et al*., 1988; Stevenson *et al*., 1990; Sohda *et al*., 1992). The mechanism by which englitazone and other agents correct glucose and insulin levels has not been fully elucidated though actions on the peroxisome proliferator-activated receptor (PPAR), which can modulate adipocyte gene-expression, may be crucial (Lehmann *et al*., 1995; Devos *et al*., 1996). Clinical trials have shown that one of the thiazolidinediones, troglitazone, is effective in the treatment of NIDDM patients both alone (Mimura *et al*., 1994) and in combination with the sulphonylureas (Iwamoto *et al*., 1996).

Details of the effects of the thiazolidinedione agents on the electrophysiology of pancreatic β-cells are sparse although it has been suggested that they act by a different mechanism to the anti-diabetic sulphonylureas (Masuda *et al*., 1995). The sulphonylureas specifically inhibit ATP-sensitive K^+ (K_{ATP}) channel function and are widely used in the treatment of NIDDM (Sturgess *et al*., 1985). They are believed to mediate their effects upon the K_{ATP} channel via a high affinity sulphonylurea receptor (SUR) present in the membrane of insulin secreting cells (Panten *et al*., 1989). The K_{ATP} channels in turn play a central role in the control of insulin secretion as their closure leads to membrane depolarization, activation of voltage-dependent Ca^{2+} channels and ultimately the exocytotic release of insulin (Ashford, 1990; Ashcroft & Rorsman, 1991). Thus, the emergence of a novel class of anti-diabetic agent which apparently acts through a different mechanism is of considerable interest.

However, in a recent study, troglitazone has been demonstrated to stimulate insulin release in isolated β-cells and the HIT insulin-secreting cell line and displace [^{3}H]-glibenclamide binding (Masuda *et al*., 1995). These investigators also showed that troglitazone failed to inhibit K_{ATP} channels, though only a single concentration of the drug was tested. In contrast, a more recent investigation has shown that troglitazone can inhibit K_{ATP} channels in the CRI-G1 insulin-secreting cell line (Lee *et al*., 1996a). We therefore decided to investigate the action of englitazone on cation conductances in the insulin-secreting CRI-G1 cell line by use of the patch clamp technique. The aim was to determine whether K_{ATP} and/or other ion channels present in this cell line (Sturgess *et al*., 1987; Kozlowski *et al*., 1991) were affected by this type of anti-diabetic agent. We present data showing that englitazone blocks K_{ATP} channels

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and Ca^{2+}-activated non-selective cation channels (NS_{Ca}) equipotently and discuss these findings in relation to previous research on the thiazolidinediones.

Methods

Preparation of CRI-G1 cells

CRI-G1 insulin secreting cells were cultured and passaged at 3–4 day intervals as previously described (Carrington *et al.*, 1986). Cells for patch-clamp studies were plated onto 3.5 cm petri dishes (Falcon 3001) at a density of approximately 1.5×10^5 cells per dish and were used 2–4 days (inclusive) after plating.

Electrical recording and analysis

This study employed both cell-free and whole-cell configurations of the patch clamp recording technique (Hamill *et al.*, 1981). Recording electrodes, when filled with electrolyte, had resistances between 8–15 MΩ for isolated patch experiments and 2–5 MΩ for whole-cell recording. Current recordings were made with an Axopatch 2D or List EPC-7 patch clamp amplifier and stored on magnetic digital audio tape (Sony DTC-1000ES) for later reproduction of figures and analysis. Single channel current analysis was determined off-line with PAT 6.2 (J. Dempster, University of Strathclyde, U.K.). Briefly, data segments between 30 and 90 s duration were filtered at 1.0 kHz by a 8-pole Bessel filter and digitized at 5.0 kHz with a Data Translation 2801A interface. The average channel activity ($N_f.P_o$) where N_f is the number of functional channels in the patch and P_o is the open probability was determined by measuring the total time spent at each unitary current level and expressed as a proportion of the total time recorded (Dempster, 1988). Changes in $N_f.P_o$ as a result of drug application are expressed as a percentage of control.

To obtain whole-cell K_{ATP} channel currents, cells were clamped at a holding potential of −70 mV and ±10 mV pulses of 200 ms duration were applied at 2 s intervals. Drug effects were quantified by measuring the amplitude of the current responses (I) during drug exposure and comparing them with those observed under control conditions (I_c). Values for the controls were obtained by calculating the mean amplitude before and after drug application. However, with concentrations of englitazone of 50 μM and above the inhibition was not easily reversed so control values were taken from the current amplitude before 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.

The effect of englitazone on whole-cell, voltage-activated currents was assessed by applying step depolarizations in 10 mV increments from a holding potential of V_h of −60 mV to +40 mV. Macroscopic voltage-activated currents were evoked by use of the computer programme 'VGEN' (J. Dempster, University of Strathclyde, U.K.). Thus, inward Na^+ currents were evoked by applying depolarizing voltage pulses, 50 ms in duration at a frequency of 1 Hz. Ca^{2+} currents and delayed outward K^+ currents were generated in response to voltage steps, 200 ms in duration at frequencies of 0.2 and 1 Hz, respectively. The frequency of stimulation did not affect the amplitude of K^+, Na^+ and Ca^{2+} currents. Voltage-activated currents were analysed and leak substracted by use of the programme 'VCAN' as previously described (Kozlowski & Ashford, 1991).

All electrophysiological experiments were performed at room temperature (19–24°C).

Drugs and solutions

The chemicals used were: englitazone sodium (Pfizer); adenosine-5′-triphosphate (dipotassium salt) (ATP), glibenclamide, trypsin (Type XI), tetraethylammonium chloride (TEA), tetrodotoxin (TTX) (Sigma); ciclazindol (Wyeth). Glibenclamide was made up as a 10^{-2} M stock solution in methanol. Englitazone sodium and ciclazindol were prepared as 10^{-2} M stock solutions in dimethysulphoxide (DMSO). DMSO (1%) and methanol (1%) were without effect on channel activity or current amplitude.

For electrophysiological studies the cells were first washed with solution A which consisted of (in mM): NaCl 135.0, KCl 5.0, $CaCl_2$ 1.0, $MgCl_2$ 1.0, HEPES 10.0 (pH 7.2 with NaOH). For whole-cell voltage clamp of the K_{ATP} channel currents, the cells were bathed in solution A while the pipette contained either solution B (in mM): KCl 140.0, $CaCl_2$ 2.0, $MgCl_2$ 1.0, K-EGTA 10.0, HEPES 10.0, pH 7.2 with KOH (resulting in free Ca^{2+} and Mg^{2+} concentrations of 25 nM and 0.65 mM, respectively) or solution C (in mM): KCl 140.0, K-EDTA 10.0, $CaCl_2$ 4.6, HEPES 10.0, pH 7.2 with KOH (resulting in free

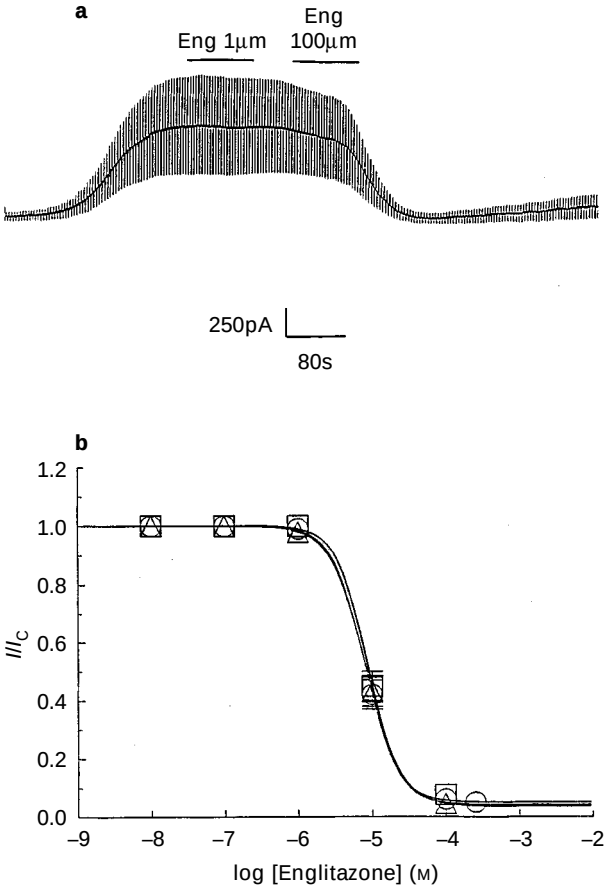

Figure 1 The effect of englitazone upon whole-cell K_{ATP} channel currents. (a) The cell membrane was clamped at −70 mV and ±10 mV pulses were applied for 200 ms every 2 s. Application of 1 μM englitazone to the bath produced no significant inhibition but the subsequent application of 100 μM englitazone was associated with a poorly reversible inhibition of K_{ATP} channel currents (as denoted by the decrease in size of the vertical lines) with an associated shift in the holding current (denoted by the horizontal line). (b) Concentration-inhibition curves for englitazone. The inhibition curves were obtained by use of recording pipettes containing 140 mM KCl with (△) <5 nM Mg^{2+}, (□) 0.65 mM Mg^{2+} and (○) 0.65 mM Mg^{2+} plus 100 μg ml^{-1} trypsin (type XI). Data are presented as fraction of the control current (I_c) taking rundown into consideration. All points are the mean of between three and five separate experiments; vertical lines show s.e.mean and where no line is apparent, the s.e.mean was smaller than the associated symbol. The curves were obtained by non-linear regression.

Ca^{2+} and Mg^{2+} concentrations of ~25 nM and <5 nM, respectively). To isolate delayed outward currents, cells were bathed in solution A to which $CdCl_2$ (1 mM) and tetrodotoxin (TTX, 300 nM) had been added whilst the pipette contained (in mM): KCl 140.0, $CaCl_2$ 2.0, $MgCl_2$ 2.5, ATP 2.0, K-EGTA 10.0, HEPES 10.0, pH 7.2 with KOH (solution D). For isolation of total inward Na^+ and Ca^{2+} current, cells were bathed in solution A while the pipette contained solution E (in mM): CsCl 140.0, $CaCl_2$ 2.0, $MgCl_2$ 1.0, Cs-EGTA 10.0, HEPES 10.0, pH 7.2 with CsOH. To study the effects of englitazone on Ca^{2+} currents, cells were bathed in solution A which also contained TTX (300 nM), TEA (10 nM) while the pipette contained solution E.

In experiments with the inside out patch configuration, the pipette contained solution F (in mM): KCl 140.0, $CaCl_2$ 1.0, $MgCl_2$ 1.0, HEPES 10.0, pH 7.2 with KOH while the bath contained solution B (Mg^{2+} present), solution C (Mg^{2+}-free) or solution F (high Ca^{2+} present to activate non-selective cation channels). For outside out patch recordings, solution B was the pipette solution while solution E was in the bath.

Statistical analysis

All data in the text are presented as the mean ± s.e.mean of the indicted number of experiments (n). The statistical significance between experimental groups was assessed by one way analysis of variance (ANOVA).

Results

Whole cell studies

K_{ATP} currents To quantify the effect of englitazone upon K_{ATP} channel currents, the whole-cell recording configuration was used as described in the Methods. Upon achieving whole-cell voltage-clamp under these conditions (i.e. with solution B with 0.65 mM Mg^{2+} ions and no ATP in the pipette) there was a gradual increase in the K^+ current at ATP was reduced in the cell due to dialysis of the cell contents with the pipette solution. This reflected the increase in K_{ATP} channel activity in the cell as the intracellular ATP concentration decreased (see Figure 1a). After several minutes the current amplitude peaked and then gradually fell, a phenomenon known as run-down (Kozlowski & Ashford, 1990). However, the addition of drugs at the time of the peak current enabled the effects of potential modulatory agents on K_{ATP} currents to be examined. Under these conditions K_{ATP} channel currents were inhibited reversibly by low concentrations (<20 μM) but irreversibly at higher concentrations of englitazone (Figure 1a). From the concentration-inhibition curve englitazone was found to have a half-maximal inhibitory concentration (IC_{50}) of 8.1 ± 0.2 μM (n = 5) with an associated Hill coefficient of 1.8 ± 0.2 (Figure 1b). Previous studies have shown that the removal of Mg^{2+} ions from the intracellular aspect of the membrane or trypsinization of the intracellular surface of the membrane can functionally or physically uncouple the sulphonylurea receptor from K_{ATP} channels (Lee *et al*., 1994a, b). Therefore the effects of englitazone on the whole-cell K_{ATP} current were re-examined but now with either the Mg^{2+}-'free' solution C (< 25 nM Ca^{2+} and <5 nM Mg^{2+}) or solution B, containing 100 μg ml^{-1} trypsin, in the patch pipette. The whole-cell K^+ currents were larger than those obtained in the presence of Mg^{2+} ions and ran down at a much slower rate, in agreement with previous studies (Lee *et al*., 1994a, b). These manipulations prevented inhibition of K_{ATP} channel currents by tolbutamide but did not influence the concentration-dependent inhibition of K_{ATP} currents by englitazone. The calculated IC_{50} values and associated Hill coefficients were 7.8 ± 0.2 μM (n = 5) and 7.7 ± 0.2 μM (n = 3), and 1.9 ± 0.1 and 2.2 ± 0.1 in the presence

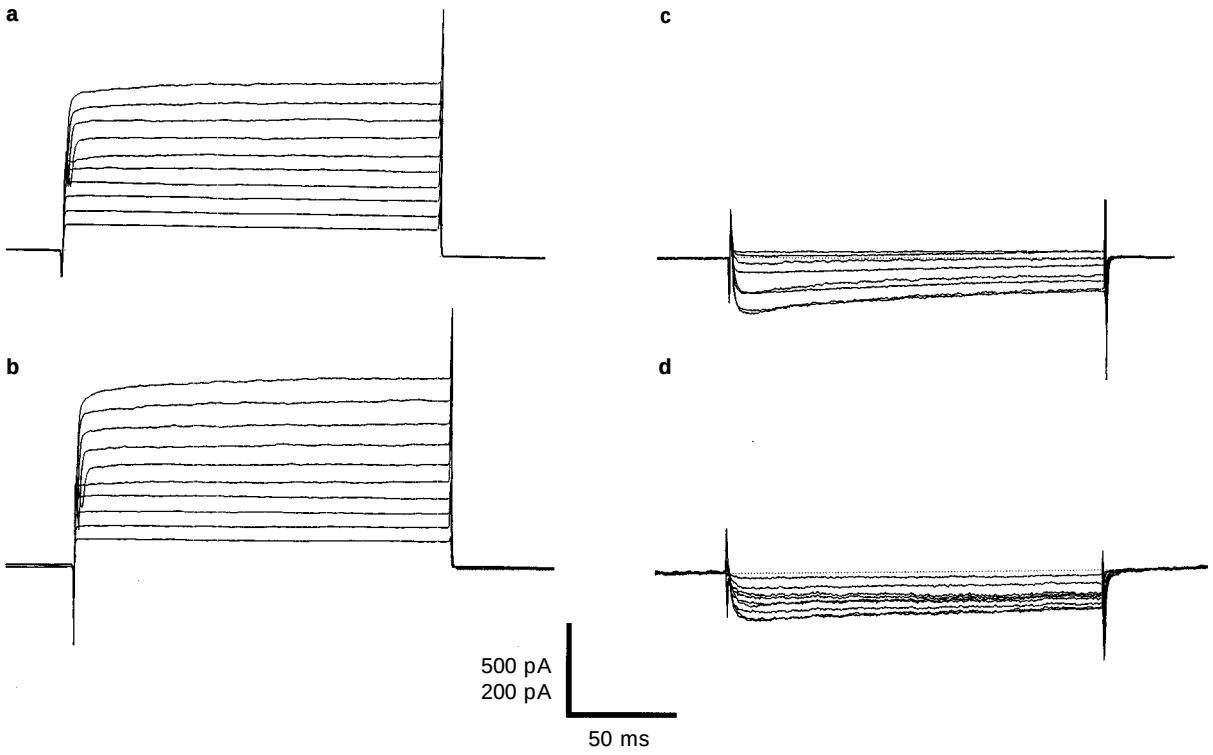

Figure 2 The effects of a high concentration of englitazone (50 μM) on voltage-activated whole cell currents. Voltage-clamp recordings were made in the whole-cell configuration with the cells held at −80 mV and then stepped to +20 in 10 mV increments. (a and b) Outward currents; bath contained solution A and the pipette contained solution D. (a) Control current; (b) upon addition of 50 μM englitazone there was a slight (13%) increase in the outward K^+ current. TTX was excluded from the bathing solution and the fast inward Na^+ currents can be made out at the start of the voltage steps and these were unaffected by the addition of englitazone. (c and d) Inward currents. (c) Control currents with 140 mM CsCl in the electrode and 10 mM TEA in the bath solution to block K^+ currents. TTX was excluded from the bathing solution. (d) Upon addition of 50 μM englitazone there was a 30% inhibition of the inward Ca^{2+} current. The current scale bar for (a) and (b): 500pA; for (b) and (d): 200pA.

of Mg^{2+}-'free' and trypsin-containing pipette solutions, respectively (Figure 1b).

Voltage-activated currents In order to determine whether englitazone, like the sulphonylureas exhibits specificity of action for K_{ATP} channels, the effects of 10 and 50 μM englitazone were examined on voltage-activated currents in CRI-G1 cells. Under conditions where both Na^+ and Ca^{2+} entry were blocked, 10 μM and 50 μM englitazone had no significant effect on the magnitude of the delayed outward K^+ current ($n=4$ for each; $P>0.05$, Figure 2) or on the time to peak of the currents and their activation threshold. In contrast, the amplitude of the voltage-activated total inward current (obtained when TTX was not included in the bathing solutions) carried by Na^+ and Ca^{2+} ions was significantly reduced by $32.6\pm3.4\%$ upon the addition of 50 μM englitazone ($n=3$; $P<0.05$, Figure 2), though 10 μM englitazone was without effect ($n=3$). However, in one further cell 50 μM englitazone in the presence of 10 μM TTX did inhibit the inward Ca^{2+} currents by 80%. The inward Na^+ currents observed in conjuction with outward K^+ currents or inward Ca^{2+} currents were not siginificantly affected by the application of 10 μM or 50 μM englitazone ($n=3$ for each, Figure 2). The Na^+ currents were identified by their rapid time course of activation and inactivation as has previously been described in the CRI-G1 cell line (Kozlowski & Ashford, 1991; Kozlowski *et al*., 1991).

Single channel studies

K_{ATP} channels In order to confirm that the reduction in the K^+ current seen in whole-cell recordings in the presence of englitazone was due to block of K_{ATP} channels a series of single channel recordings were undertaken. In the initial experiments the concentration-dependent effects of englitazone on inside-out excised membrane patches were examined in the presence and absence of Mg^{2+} ions. When applied in the bathing solution to the intracellular surface of membrane patches excised from CRI-G1 cells, englitazone (20 μM) reversibly inhibited single K_{ATP} channel activity both in the presence of 0.65 mM Mg^{2+} and when the level of free Mg^{2+} ions had been reduced to less than 5 nM (Figure 3). The inhibition of channel activity was concentration-dependent with calculated IC_{50} values obtained of 10 ± 4 μM ($n=3$) and 13 ± 5 μM ($n=4$) and Hill slopes of approximately 2 in the 0.65 mM and 5 nM Mg^{2+} bathing solutions, respectively. This reduction in channel activity was not associated with a change in the single channel conductance, over the range 0 mV to -50 mV, being 56 ± 4 pS in control ($n=3$) and 55 ± 5 pS in the presence of 20 μM englitazone ($n=3$), nor was the inhibition of P_o voltage-dependent over this range. Essentially identical results were obtained when englitazone was added to the intracellular surface of patches which had previously been treated with 100 μg ml^{-1} trypsin ($n=4$), which results in the functional

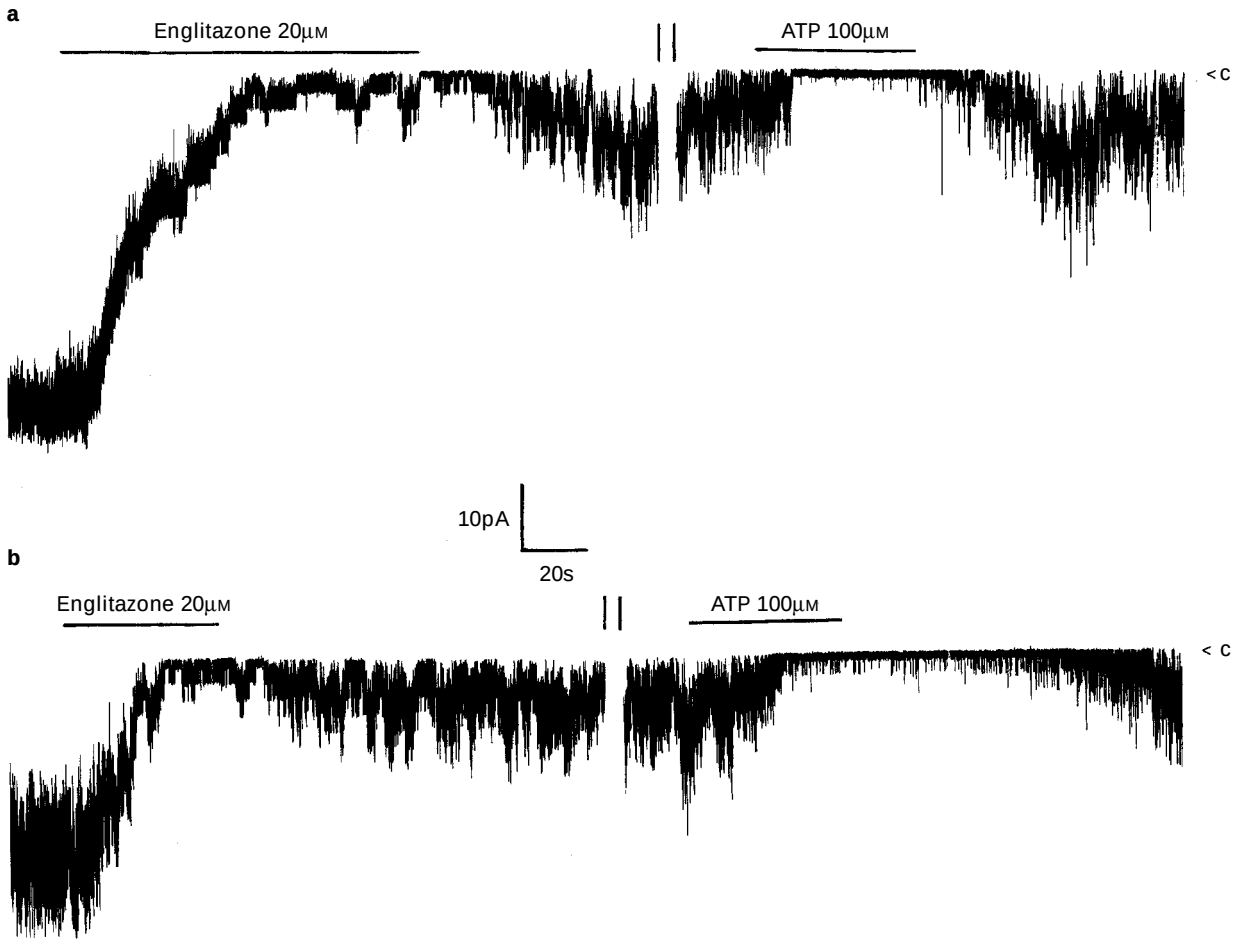

Figure 3 The effects of englitazone on K_{ATP} channels in the presence and absence of Mg^{2+} ions at the intracellular surface of excised inside out patches. Representative traces of (a) multi-channel currents recorded at -40 mV in the presence of 0.65 mM Mg^{2+} and (b) multi-channel currents recorded at -45 mV in the presence of <5 nM Mg^{2+}. Application of 20 μM englitazone produced a marked inhibition of channel activity in both Mg^{2+} concentrations which was partly reversed on washout of drug. The subsequent application of 100 μM ATP reversibly blocked channel activity in each case. The N_fP_o values were as follows: (a) control 23.22; 20 μM englitazone 1.82; wash 3.46; 100 μM ATP 0.19; wash 2.27. (b) Control 10.07; 20 μM englitazone 0.71; wash 2.51; 100 μM ATP 0.03; wash 2.96. The parallel vertical bars above the traces indicate breaks of approximately 30 s during the wash out of englitazone.

uncoupling of the sulphonylurea receptor from the K_{ATP} channel (Lee *et al*., 1994b) (Figure 4).

As with the whole cell recordings it was clear that concentrations of englitazone of 20 μM and above produced inhibition of K_{ATP} channels which was only partly reversed upon wash out of the drug (Figures 3 and 4). Though the channel activity did not recover fully, even allowing for the different rates of channel run-down in the presence or absence of Mg^{2+} ions, those K_{ATP} channels still active retained their ATP sensitivity ($n=2$ to 5 for each; with Mg^{2+}, Mg^{2+}-free and with trypsin; see Figures 3 and 4). Furthermore the inhibitory effect of 100 μM ATP on K_{ATP} channel activity was unchanged in the concomitant presence of 1 μM englitazone ($n=3$).

Englitazone also inhibited K_{ATP} channel activity when applied to the extracellular aspect of outside-out membrane patches. For example application of 20 μM englitazone produced $68\pm5\%$ ($n=3$) inhibition of K_{ATP} channel activity under these conditions, which compares well with the $72\pm7\%$ ($n=5$) inhibition produced by this concentration of englitazone applied to the cytoplasmic surface of inside-out membrane patches.

Ca^{2+}-activated non-selective cation channels Another potential site of action for englitazone in the CRI-G1 insulin-secreting cell line is the Ca^{2+}-activated non-selective cation (NS_{Ca}) channel (Sturgess *et al*., 1987; Reale *et al*., 1995). NS_{Ca} channel activity was induced in inside-out excised patches by the addition of 1 mM free Ca^{2+} ions to the bathing solution. This relatively high concentration of Ca^{2+} ions at the cytoplasmic surface of the membrane produced at least half-maximal activation of the NS_{Ca} channels in CRI-G1 cells (Sturgess *et al*., 1987). The addition of 1 mM Ca^{2+} ions to the bath evoked rapid run-down and/or inhibition of K_{ATP} channels followed by a more gradual activation of lower conductance NS_{Ca} channel activity (Figure 5). The NS_{Ca} channels had a mean single channel conductance of 26 ± 4pS ($n=5$) in symmetrical 140 mM KCl (voltage range ±80 mV) and channel activity was voltage-dependent (Figure 5), increasing with depolarization of the membrane in agreement with the previous study (Sturgess *et al*., 1987).

The addition of englitazone to the intracellular surface of inside-out patches under these conditions produced reversible inhibition of NS_{Ca} channel activity (Figure 6). The inhibition produced by englitazone was concentration-dependent with a calculated IC_{50} value of 9.7 ± 4.1 μM ($n=3$) and an associated Hill coefficient of 1.4 ± 0.4. The englitazone-induced inhibition was achieved by reducing the channel activity while the conductance remained unchanged at 25 ± 5 pS (range ±80 mV) ($n=3$). The reduction in channel activity was not significantly voltage-dependent as 20 μM englitazone produced $76\pm7\%$ and $74\pm6\%$ inhibition at $+50$ mV and -50 mV, respectively ($n=3$ for each). The NS_{Ca} channels retained their ATP-sensitivity after englitazone treatment as the addition of 100 μM ATP to the bath almost completely abolished channel activity ($n=6$) (see Figure 6). The inhibition of NS_{Ca} channel activity by relatively high concentrations of englitazone (>20 μM) was also difficult to reverse

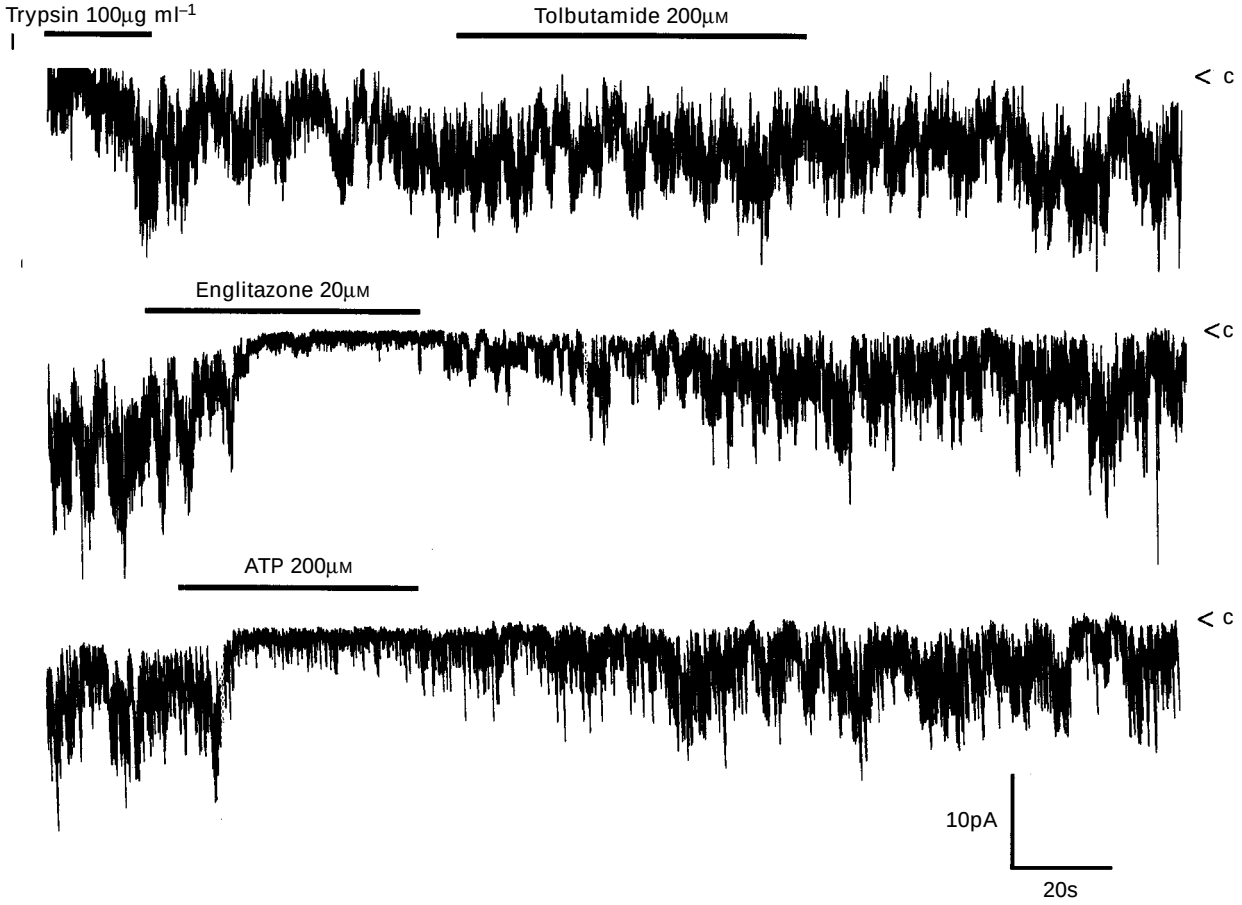

Figure 4 The effects of englitazone on K_{ATP} channels after trypsinization of the intracellular surface of an excised inside out patch. Multi-channel currents recorded at -40 mV in the presence of 0.65 mM Mg^{2+}. Application of 100 μg ml^{-1} trypsin caused an increase in K_{ATP} channel activity which was unaffected by the addition of 200 μM tolbutamide. Application of 20 μM englitazone produced a marked inhibition of channel activity which was partly reversed on washout of drug. The subsequent application of 200 μM ATP reversible blocked channel activity. The N_fP_o values were as follows: control 2.85; after trypsin 5.72; 200 μM tolbutamide 5.64; wash 6.27; 20 μM englitazone 0.05; wash 4.61; 200 μM ATP 0.12; wash 3.36.

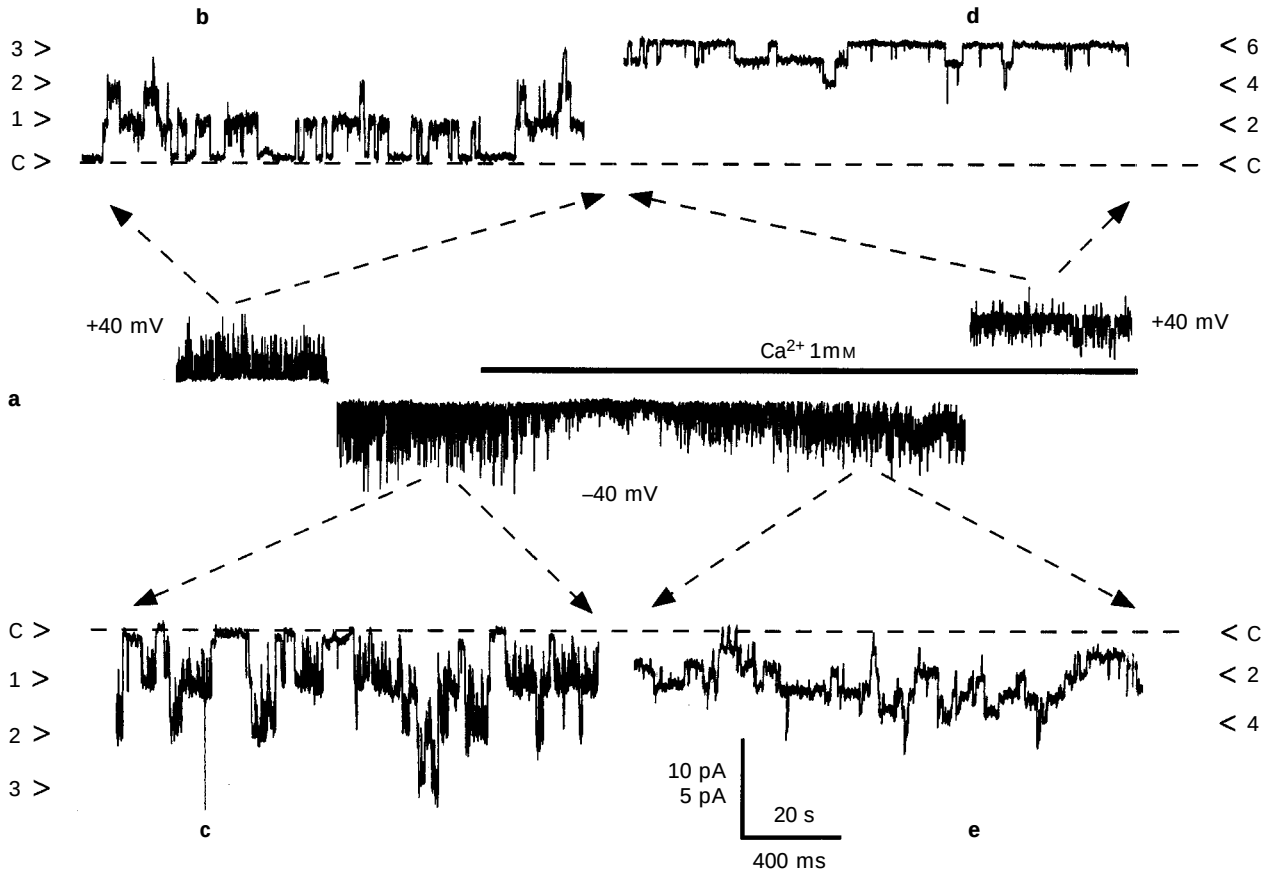

Figure 5 The effect on ion channel activity of the addition of 1 mM Ca^{2+} to the intracellular surface of an excised inside-out patch. The central trace (a) was a continuous multi-channel recording in symmetrical 140 mM KCl at +40 mV and −40 mV showing the gradual reduction in K_{ATP} channel activity upon application of 1 mM Ca^{2+} and the subsequent activation of NS_{Ca} channels. The other traces (b, c, d, e) are expanded sections of this recording. (b) and (c) K_{ATP} channel activity at +40 mV and −40 mV before the addition of 1 mM Ca^{2+} with N_fP_o values of 0.44 and 0.32 and conductances of 42pS and 53pS, respectively. (d) and (e) Examples of the NS_{Ca} activity recorded in the presence of 1 mM Ca^{2+} at +40 mV and −40 mV with N_fP_o values of 5.9 and 2.1 with single channel conductances of 25pS and 23pS, respectively.

but this was less marked compared to K_{ATP} channel activity perhaps due to the lack of rundown of NS_{Ca} channels (Sturgess *et al.*, 1987; 1988; Reale *et al.*, 1995).

These data clearly show that englitazone can inhibit two different types of ATP-regulated ion channels in CRI-G1 cells, the K_{ATP} and the NS_{Ca} channel, with similar potency. Previous investigations have shown that the NS_{Ca} channel is insensitive to sulphonylureas (Sturgess *et al.*, 1988). Consequently englitazone may act via a non-SUR site to induce inhibition of K_{ATP} channel activity. Thus, both NS_{Ca} and K_{ATP} channel structures may contain an inhibitory binding site, independent from the SUR, which can mediate a reduction in channel activity. Recently ciclazindol, an anorexic agent, has also been shown to block K_{ATP} channel activity under conditions where the SUR and K_{ATP} channel have been functionally uncoupled (Lee *et al.*, 1996b). This agent, a non-sulphonylurea, inhibits K_{ATP} with an IC_{50} of 40 nM. However, in excised inside-out patches application of ciclazindol at 20 μM caused only a slight reduction in NS_{Ca} channel activity. The inhibition appeared to be different from that caused by englitazone in that the block was 'flickery' (Figure 7) and voltage-dependent, with a greater reduction in channel current amplitude at depolarized potentials. For example, 20 μM ciclazindol caused $24 \pm 4\%$ and $13 \pm 3\%$ inhibition of channel activity at potentials of +50 mV and −50 mV, respectively ($n = 5$ for each).

Discussion

These results demonstrate that englitazone inhibits K_{ATP} channel activity in the CRI-G1 insulin-secreting cell line, producing half-maximal channel inhibition at 8 μM. This compares well with the first generation antidiabetic sulphonylurea, tolbutamide, which inhibits K_{ATP} channel currents in this cell line with an IC_{50} of 12 μM (Lee *et al.*, 1994a), but it is clearly less effective than K_{ATP} channel inhibitors such as glibenclamide (a second generation sulphonylurea, IC_{50} = 2.1 nM; Lee *et al.*, 1994a) or ciclazindol (an anorexic agent, IC_{50} = 40 nM; Lee *et al.*, 1996b. However, the mechanism by which englitazone blocks K_{ATP} channel activity appears to differ from the sulphonylureas as the effect is independent of intracellular Mg^{2+} ion concentration and is retained after trypsinization of the cytoplasmic surface of the cell membrane, treatments which functionally uncouple the SUR from K_{ATP} channels (Lee *et al.*, 1994a, b).

An intriguing result of this study was the block by englitazone of the Ca^{2+}- activated non-selective cation channel (NS_{Ca}). The role of NS_{Ca} channels in insulin secreting cells has yet to be fully elucidated. However, a Ca^{2+}- and cyclicAMP-dependent non-selective cation (I_{cAMP}) current consistent with NS_{Ca} channel activity has been identified in isolated pancreatic β-cells and βTC6 and HIT-T15 insulinoma cell lines (Holz *et al.*, 1995). The authors show this non-selective current to be regulated by glucagon-like peptide-1 (GLP-1) and suggest that it may play an important role in the regulation of insulin secretion. Therefore, englitazone could prove useful in the development of selective blockers of non-selective cation channels and may help identify the functions of this channel in insulin-secreting cells or similar channels in other cells (Yau, 1994). It was notable that the concentration-dependence of block of the NS_{Ca} and K_{ATP} channels were almost identical and that both were voltage-independent. This raises the possibility

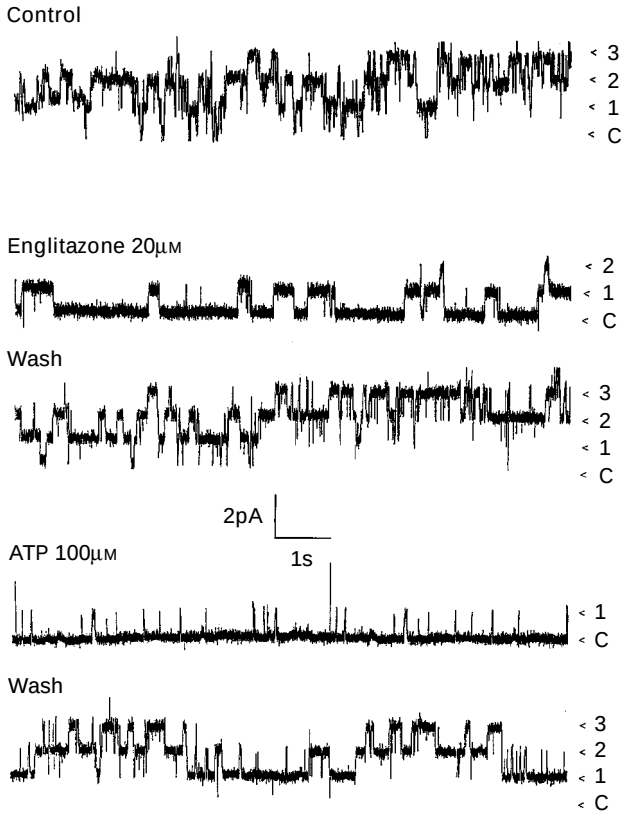

Figure 6 The effect of englitazone on NS_{Ca} channel activity. An inside out patch clamp recording at +50 mV in symmetrical 140 mM KCl. The application of 20 μM englitazone caused a reversible inhibition of NS_{Ca} channel activity. Subsequent addition of 100 μM ATP also caused reversible blockade of channel activity. N_fP_o values were as follows: control 2.64; 20 μM englitazone 0.09; wash 1.59; 100 μM ATP 0.006; wash 1.45.

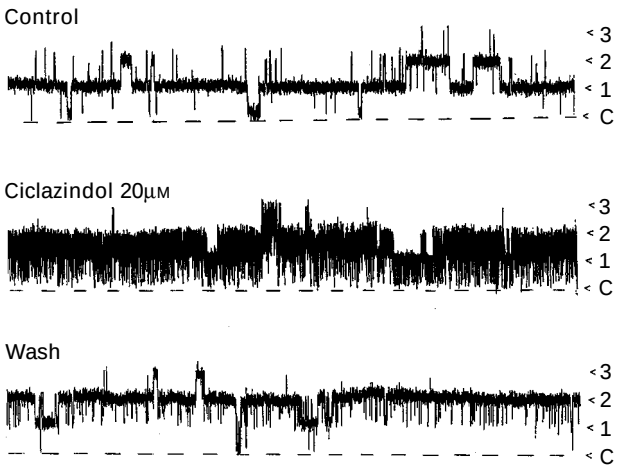

Figure 7 The effect of ciclazindol on NS_{Ca} channel activity. An inside-out patch clamp recording at +50 mV in symmetrical 140 mM KCl. The application of 20 μM ciclazindol caused a reversible 'flickery' blockade of NS_{Ca} channel activity. N_fP_o values were as follows: control 1.71; 20 μM ciclazindol 1.28; wash 1.66.

that there is some homology in the site of action on both types of ion channel. As the identity of the components which make up the β-cell K_{ATP} channel are still unclear (Aguilar-Bryan *et al*., 1995; Inagaki *et al*., 1995a, b; Ammala *et al*., 1996) and the sequence of the NS_{Ca} channel in CRI-G1 cell has yet to be determined, further research is required to resolve this question.

Although ciclazindol, like englitazone, inhibits K_{ATP} channels functionally uncoupled from the SUR at nM concentrations (Lee *et al*., 1996b), it has little effect on NS_{Ca} channels at this concentration range. Much higher concentrations (e.g. 20 μM) induce a voltage-dependent reduction in channel activity with a concomitant flickery block of open channels and reduction in amplitude consistent with a fast open channel block (Hille, 1992). Thus englitazone likely acts at a site separate from that of ciclazindol on both channel types.

Our data demonstrating that englitazone blocks K_{ATP} channels appears to contradict previous findings. Animal studies have shown that englitazone and other thiazolidinediones act as antidiabetic agents by enhancing the action of insulin without stimulating insulin secretion from the pancreas (Fujita *et al*., 1988; Stevenson *et al*., 1990; Sohda *et al*., 1992). Englitazone by inhibiting K_{ATP} channel activity would be expected to depolarize pancreatic β-cells and increase insulin release (Ashford, 1990; Ashcroft & Rorsman, 1991). Furthermore, recent studies with troglitazone have shown that insulin secretion in isolated pancreatic islets and HIT cells is stimulated by concentrations of 1 μM and 10 μM but inhibited by higher (100 μM) concentrations (Masuda *et al*., 1995). Therefore in isolated pancreatic tissue or β-cell lines it is possible to elicit an increase in insulin secretion upon the addition of a thiazolidinedione antidiabetic. A further complication is that troglitazone has been shown to have a non-competitive binding site at or near the SUR and that it does not block K_{ATP} channels (Masuda *et al*., 1995). These authors show that sulphonylurea binding was displaced by troglitazone but with an IC_{50} of between 50 and 76 μM, considerably higher concentrations than those required to stimulate insulin release and glucose uptake. These data support the hypothesis that troglitazone interacts with the SUR but this is not part of the mechanism by which the drug stimulates insulin release. In their patch clamp study of K_{ATP} channels troglitazone was added at a single concentration of 1 μM, below that which maximally stimulates insulin release in the same cells. Therefore, it is possible that the inhibitory effect of troglitazone on K_{ATP} channels was missed and would be obvious at concentrations above 1 μM, particularly as troglitazone has an apparent EC_{50} for insulin release from islets in the micromolar range (Masuda *et al*., 1995) and could well have a Hill slope greater than one, as is the case for englitazone (see Results section). This is probably the case, as troglitazone has recently been shown to inhibit K_{ATP} channel activity in the CRI-G1 cell line with an IC_{50} of 0.7 μM and to be capable of depolarizing the cell membrane (Lee *et al*., 1996a).

Englitazone has been shown to enhance insulin action but not insulin release in normal rats and NIDDM rodent models (Fujita *et al*., 1988; Stevenson *et al*., 1990; 1991). This is perhaps surprising in view of its ability to inhibit K_{ATP} channel activity over the same concentration range. A number of possible explanations may exist: englitazone may inhibit other ion channel components of the insulin release mechanism in β-cells; the enhancement of peripheral insulin action may be sufficient to initiate feedback mechanisms to limit insulin release (Fujita *et al*., 1988; Kreutter *et al*., 1990; Sohda *et al*., 1992); or englitazone may influence other ATP-modulated ion channels (Ashford *et al*., 1990; Rowe *et al*., 1996) present in CNS glucose-sensing neurones involved in the central control of metabolism (Luiten *et al*., 1987) which, through peripheral feedback mechanisms, result in restriction of pancreatic insulin release.

However, it is unlikely that englitazone blocks voltage-gated cation channels resulting in inhibition of insulin release as voltage-gated K^+ channels were unaffected by the drug, while Ca^{2+} currents were inhibited only at a high concentration (50 μM). The Na^{2+} currents appeared unaffected by englitazone even at 50 μM, though a Na^+ component may be present in the inward currents inhibited by englitazone (Figure 2), the time course of the current was consistent with it being identified as a Ca^{2+} current (Kozlowski & Ashford, 1991; Kozlowski *et al*., 1991). These observations do not rule out the

possibility that higher concentrations of englitazone may inhibit voltage-gated ion channels significantly and this may be relevant to the inhibition of insulin release for 100 μM troglitazone (Masuda *et al*., 1995).

The NS_{Ca} channel may be a likely site of action for englitazone resulting in disruption of insulin release. The activation of I_{cAMP} in insulinoma and β-cells causes membrane depolarization and increased intracellular Ca^{2+} levels and may influence insulin-secretion (Holz *et al*., 1995). Blockade of NS_{Ca} channels, which may underlie I_{cAMP}, would limit the membrane depolarization and rise in intracellular Ca^{2+} concentration and so inhibit the release of insulin. Thus as englitazone blocks K_{ATP} and NS_{Ca} channels equipotently the net effect on insulin release may be minimal. Consequently, the overall effect of thiazolidinediones on insulin secretion may reflect the relative importance of K_{ATP} versus NS_{Ca} channels in this process and the degree of inhibition of each produced by therapeutic concentrations of these drugs. Estimates of therapeutic concentrations of the thiazolidinediones appear to place them in the low micromolar range (Masuda *et al*., 1995; Stevenson *et al*., 1995). So it may be the case that the insulin-sensitizing effects of these drugs occur at concentrations below that at which they can inhibit channel activity. However, chronic application of 1 μM englitazone may also inhibit channel activity and this should be addressed in further experiments.

That englitazone does not produce an increase in plasma insulin even though it blocks K_{ATP} channels may also be due to its effects on peripheral insulin resistance (Fujita *et al*., 1988; Stevenson *et al*., 1991; Mimura *et al*., 1994). The thiazolidinediones, including englitazone, potentiate insulin actions in a variety of peripheral tissues (Fujita *et al*., 1988; Kreutter *et al*., 1990) and stimulate glucose uptake in adipocytes (Kreutter *et al*., 1990). Therefore, though thiazolidinediones can stimulate insulin release in isolated β-cells (Masuda *et al*., 1995), the marked changes in peripheral insulin sensitivity and glucose uptake allow normal glucose levels to be maintained by reduced plasma insulin concentrations in normal controls (Stevenson *et al*., 1991) and maintain insulin levels in the diabetic state (Stevenson *et al*., 1990); Mimura *et al*., 1994). The peripheral changes in insulin resistance may be sufficient to allow the thiazolidinediones to control glucose and insulin levels without recourse to any central mechanism. However, englitazone may also effect the glucose-sensing neurones of the CNS which influence metabolism through autonomic and neuroendocrine output (Luiten *et al*., 1987). Therefore, an investigation of the effects of the thiazolidinediones on central glucose-sensing neurones may prove worthwhile.

The finding that englitazone blocks K_{ATP} channels in CRI-G1 cells at a site separate from that utilized by the sulphonylureas will require further investigation. The use of englitazone or other thiazolidinedione derivatives on cloned versions of the K_{ATP} channel-SUR complex may reveal the exact location of this inhibitory site. Similarly, the novel action of this drug on the NS_{Ca} channel may help our understanding of the function of this channel and its structural relationship to K_{ATP}. These studies in combination with the investigation of the effects of antidiabetic thiazolidinediones on other peripheral and central K_{ATP} and NS_{Ca} channels may help in refining these agents to produce more selective treatments of NIDDM.

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