Leptin activates ATP-sensitive potassium channels in the rat insulin-secreting cell line, CRI-G1

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1. Whole-cell current-clamp recordings demonstrate that leptin (0·3–10 nM) hyperpolarizes CRI-G1 insulin-secreting cells. This effect is slow on onset and is not reversed on washout of the leptin.

2. Voltage-clamp recordings indicate that leptin activates a potassium conductance in the presence of intracellular ATP (5 mM), but has no effect in its absence. Following activation of ATP-sensitive K^+ (K_{ATP}) current by diazoxide (0·2 mM), addition of leptin did not alter cell membrane potential or potassium current further.

3. The leptin-induced hyperpolarization and increased potassium conductance are completely inhibited by the application of the sulphonylureas tolbutamide (100 μM) and glibenclamide (0·5 μM).

4. Cell-attached and inside-out single-channel recordings indicate that leptin activates tolbutamide-sensitive K_{ATP} channels in CRI-G1 insulin-secreting cells.

Recent studies indicate that the *ob* gene product, leptin, is produced exclusively in adipose tissue (Masuzaki *et al.* 1995) and that in *ob/ob* mice there is a defect in the *ob* gene that results in abnormal leptin production and consequent development of severe obesity and diabetes (Zhang, Proenca, Maffei, Barone, Leopold & Friedman, 1994). The *db* gene encodes the leptin receptor, which in *db/db* mice has a point mutation resulting in alternative splicing of the coding region and the insertion of a premature termination signal, resulting in leptin insensitivity and the obese, diabetic phenotype (Lee *et al.* 1996). Consequently, leptin administration to *db/db* mice has no effect on feeding behaviour and does not normalize the metabolic status of these animals (Campfield, Smith, Guisez, Devos & Burn, 1995; Halaas *et al.* 1995; Stephens *et al.* 1995), but administration of leptin to *ob/ob* mice results in reduced body weight, percentage body fat, food intake and serum concentrations of glucose and insulin (Campfield *et al.* 1995; Halaas *et al.* 1995; Pelleymounter *et al.* 1995; Stephens *et al.* 1995). It is notable that the changes in glucose and insulin levels induced by leptin administration to *ob/ob* mice precede any effect on body weight.

Although a central hypothalamic site of action has been postulated for the effects of leptin on food intake and weight loss (Jacob *et al.* 1997), there is also considerable evidence for direct effects on peripheral tissues, including pancreatic β-cells. Leptin receptor mRNA has been shown to be expressed in rat primary pancreatic islets, the insulinoma cell line βTC-3 (Keiffer, Heller & Habener, 1996) and *ob/ob* and wild-type mouse pancreatic islets (Emilsson, Liu, Cawthorne, Morton & Davenport, 1997). The latter study also demonstrated that leptin produced a dose-dependent inhibition of glucose-stimulated insulin secretion from isolated islets of *ob/ob* but not *db/db* mice. The present study shows that leptin activates ATP-sensitive K^+ (K_{ATP}) channels to hyperpolarize CRI-G1 insulin-secreting cells, an action consistent with inhibition of insulin release.

METHODS

Cell culture

Cells from the insulin-secreting cell line CRI-G1 were grown in Dulbecco's modified Eagle's medium with sodium pyruvate and glucose, supplemented with 10% (v/v) fetal calf serum and 1% (v/v) penicillin and streptomycin at 37 °C in a humidified atmosphere of 95% O_2 and 5% CO_2. Cells were passaged at 2–5 day intervals as previously described (Carrington, Rubery, Pearson & Hales, 1986), plated onto 3·5 cm Petri dishes (Falcon 3001) and used 1–4 days after plating.

Electrophysiological recording and analysis

Experiments were performed using whole-cell current- and voltage-clamp recording configurations to monitor membrane potential and macroscopic current, respectively, with the cell-attached and outside-out configurations used to examine single-channel responses. Recording electrodes were pulled from borosilicate glass capillaries and had a resistance of 8–12 MΩ for cell-attached recordings and 1–5 MΩ for whole-cell and outside-out experiments when filled with electrolyte solution. Currents and voltages were measured

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using List EPC-7 or Axopatch 200B amplifiers. Data were recorded onto digital audio tape and replayed for illustration onto a Gould TA 240 chart recorder.

The potential across the membrane is expressed using the usual sign convention, negative inside, and all inward currents, from extra- to intracellular, are shown as downward deflections. 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 the membrane potential was held at −50 mV and 10 mV steps of 100 ms duration every 200 ms applied (range of voltages, −120 to −30 mV). In some current-clamp experiments where no voltage-clamp excursions were used, hyperpolarizing current pulses (20 pA and 0·2 s duration) were applied every 5 s to monitor input conductance changes. All values in the text are expressed as means ± S.E.M. Statistical significance between data sets was tested using Student's paired t test.

For whole-cell and outside-out experiments, the pipette solution contained (mM): 140 KCl, 0·6 $MgCl_2$, 3·95 $CaCl_2$, 5·0 EGTA, 5·0 or 1·0 ATP and 10 Hepes (pH 7·2; free [Ca^{2+}] of 1 μM), or an identical solution but with 2·73 $CaCl_2$ and 10 EGTA (free [Ca^{2+}] of 100 nM), and the bath solution consisted of normal saline (mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$ and 10 Hepes (pH 7·4). The pipette solution for cell-attached (and inside-out) single channel recordings contained (mM): 140 KCl, 1 $CaCl_2$, 1 $MgCl_2$ and 10 Hepes; pH 7·2. The data were analysed for current amplitude, average current activity ($I = N_f P_o i$, where N_f is the number of functional channels and P_o is the open state probability) and P_o, as described previously (Lee, Rowe & Ashford, 1995). All solution changes were achieved by superfusing the bath with a gravity feed system at a rate of 10 ml min^{-1}, which allowed complete bath exchange within 2 min. All experiments were performed at room temperature (22–25 °C). ATP, tolbutamide, glibenclamide and diazoxide were obtained from Sigma (UK) and tolbutamide and glibenclamide made up as 100 mM stock solutions in DMSO, and diazoxide as a stock solution in 0·1 M 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. Human recombinant leptin was supplied by Dr Peter Lind of Pharmacia-Upjohn (Stockholm, Sweden) and was prepared as a 1 μM stock solution in normal saline and further diluted in normal saline containing 0·2% (w/v) bovine serum albumin as a carrier.

RESULTS

Under current-clamp conditions with 5 mM ATP present in the electrode solution (to maintain the ATP-sensitive potassium (K_{ATP}) channels in the closed state during whole-cell perfusion of the cell interior), the mean resting membrane potential of CRI-G1 insulinoma cells was −39 ± 10·1 mV ($n = 40$). Following application of leptin (1–10 nM), all cells examined to date ($n = 42$) have responded by hyperpolarization (regardless of whether 0·1 or 1·0 μM Ca^{2+} was present in the pipette). For example, application of 10 nM leptin (Fig. 1B) resulted in CRI-G1 cell hyperpolarization to −74 ± 4·9 mV ($n = 8$), an effect not reversed following wash of the leptin from the bath ($n = 3$; after 50 min the cells were still hyperpolarized). The hyperpolarizing response to leptin is slow, taking 7–8 min before initiation of response and a further 8–10 min to achieve a maximum. This slow response is not dependent upon the concentration of leptin applied (0·3–50 nM). Control experiments using identical recording conditions but where no leptin was added to the bathing solution demonstrated that no spontaneous alterations of resting membrane potential occurred during time of recording (Fig. 1A). Examination of the voltage-clamped macroscopic currents (Fig. 1C and D) show that prior to leptin application (10 nM), the control slope conductance of the cells was 0·48 ± 0·08 nS, and following exposure to leptin a current was activated that displayed no voltage or time dependent properties, and resulted in a linear current–voltage relationship with a slope conductance of 2·7 ± 0·6 nS ($n = 6$; $P < 0·05$). Essentially identical data were obtained for membrane potential (final values of −69 ± 4·2 and −71 ± 1·7 mV) and slope conductance (final values of 3·9 ± 1·0 and 4·3 ± 0·8 nS) on application of 1 nM ($n = 5$) and 3 nM ($n = 9$) leptin, respectively. The reversal potential associated with the increase in cell conductance for all three concentrations of leptin was essentially identical, and the mean pooled value was −79 ± 0·9 mV ($n = 20$), consistent with leptin causing the activation of a potassium current (the calculated reversal potential for a pure potassium channel under the conditions of our experiments is −84 mV). The lowest concentration of leptin that induced a hyperpolarization (from −40 to −62 mV) associated with a concomitant increase in potassium conductance of these cells was 0·3 nM ($n = 1/3$ tested).

This experiment was repeated but with omission of ATP from the electrode (and therefore the cell interior), allowing the cell to hyperpolarize slowly with a concomitant increase in cell conductance as the ATP is washed out of the cell interior and K_{ATP} channels are opened (Fig. 2A). Addition of leptin to the bathing solution following run-up of the K_{ATP} conductance resulted in no significant ($P > 0·05$; paired t test) increase in total conductance; mean slope conductance following run-up was 9·0 ± 2·3 nS and in the presence of leptin (3–10 nM), the slope conductance was 10·1 ± 2·8 nS ($n = 4$). In addition, it was noted that even in the constant presence of leptin, the K_{ATP} conductance slowly decreased with time ('run-down'), resulting in a slow depolarization of the cell resting membrane potential (Fig. 2A). The hyperglycaemic agent diazoxide hyperpolarizes insulin-secreting cells by activating K_{ATP} currents (Trube, Rorsman & Ohno-Shosaku, 1986; Sturgess, Kozlowski, Carrington, Hales & Ashford, 1988), an action that is also dependent on the presence of intracellular nucleotides (Kozlowski & Ashford, 1992). Diazoxide (200 μM) stimulated an increase in K_{ATP} current with 5 mM ATP in the electrode solution (Fig. 2B) resulting in hyperpolarization of CRI-G1 cells from −39 ± 1·8 to −66 ± 1·3 mV with an associated increase in cell slope conductance from 0·78 ± 0·08 to 9·79 ± 0·95 nS ($n = 5$). The reversal potential associated with diazoxide action was −79 ± 1·0 mV ($n = 5$). Following activation of K_{ATP} current by diazoxide, leptin (10 nM) was unable ($P > 0·05$) to increase the membrane potential or slope conductance (10·20 ± 0·83 nS) further. However, the increased K_{ATP}

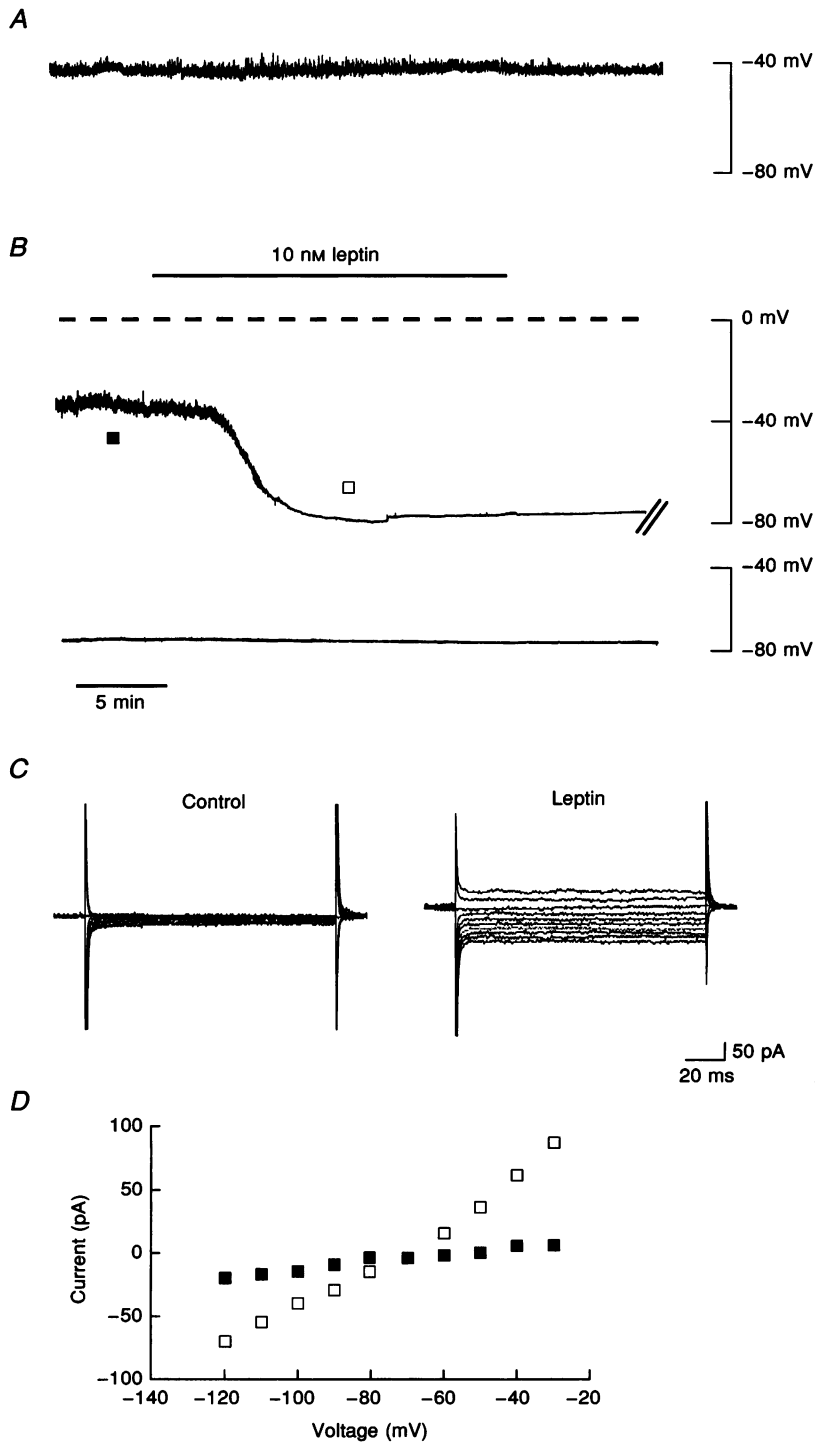

Figure 1. Effect of leptin on CRI-G1 cell membrane potential and membrane current

A, whole-cell current-clamp recording of a CRI-G1 cell following dialysis with an electrode solution containing 5 mM ATP. In the absence of leptin there is no discernable change in the resting membrane potential. In this and subsequent current-clamp figures, the trace begins approximately 5 min following formation of the whole-cell configuration. *B*, leptin (10 nM) applied for the period indicated hyperpolarized the cell from a resting membrane potential of −40 to −80 mV. This effect was not readily reversible on washout of leptin for 50 min. The break corresponds to 20 min; the traces shown are continuous. *C*, a family of membrane currents evoked in the absence and presence of 10 nM leptin, respectively. Currents were recorded under voltage-clamp at the points specified in *B*: control (■) and leptin (□) during a series of voltage steps. *D*, plot of the currents obtained in *C*, *versus* membrane potential. Leptin (□) increased the slope conductance relative to control (■), with a reversal potential of −76 mV.

current and hyperpolarization associated with the action of diazoxide was reversed, in the presence of leptin (Fig. 2*B*), by application of 100 μM tolbutamide ($n = 3$). Thus leptin is incapable of eliciting the activation of a potassium conductance at a time when the K_{ATP} current is maximal in the absence of intracellular ATP or in the presence of ATP but following stimulation by diazoxide. These data are consistent with the K_{ATP} channel as the potassium channel type activated by leptin in these cells. Further evidence to substantiate this proposal was obtained from pharmacological examination of the potassium current induced by leptin.

Following leptin-induced (3 nM) cell hyperpolarization, application of TEA (1 mM), which in insulin-secreting cells does not inhibit K_{ATP} channels at concentrations that block voltage- and calcium-gated K^+ channels (Ashcroft & Rorsman, 1991), did not cause cell depolarization ($n = 4$) consistent with K_{ATP} channel activation. However, the leptin response was sensitive to the application of the sulphonylureas, tolbutamide and glibenclamide, purported selective blockers of K_{ATP} channels. The addition of tolbutamide (100 μM) to CRI-G1 cells in the presence of 3 nM leptin (following completion of the hyperpolarization and conductance increase) induced an immediate and complete reversal of the effects of leptin (Fig. 3*A*), such that the membrane potential returned to a value indistinguishable from the membrane potential prior to leptin activation (approximately −40 mV). The leptin-induced increase in cell conductance also reverted to control values in the presence of tolbutamide (Fig. 3*B*) and the reversal potential for this action was $-77 \pm 1{\cdot}3$ mV ($n = 6$). Removal of tolbutamide elicited a complete recovery of leptin-induced hyperpolarization and conductance (Fig. 3*A*). In similar

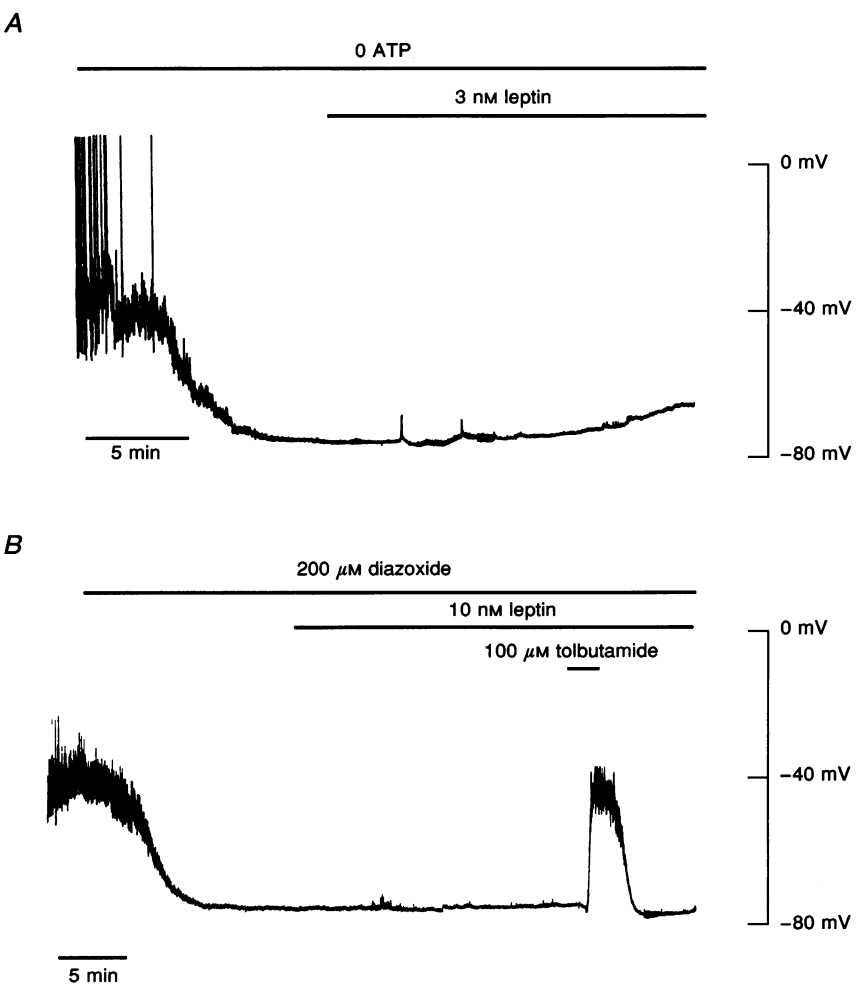

Figure 2. Lack of effect of leptin following activation of K_{ATP} channels

A, current-clamp record of a cell dialysed with a solution containing no added ATP. Following washout of ATP, the cell hyperpolarizes due to the opening of K_{ATP} channels. Addition of leptin (3 nM) following run-up of K_{ATP} channels has no effect on membrane potential. *B*, current-clamp record of membrane potential of a cell dialysed with 5 mM ATP. Application of the K_{ATP} channel activator diazoxide (200 μM) hyperpolarized the cell to −76 mV. Leptin (10 nM) applied after the diazoxide-induced hyperpolarization failed to affect the cell membrane potential. The sulphonylurea tolbutamide (100 μM) readily reversed this action of diazoxide.

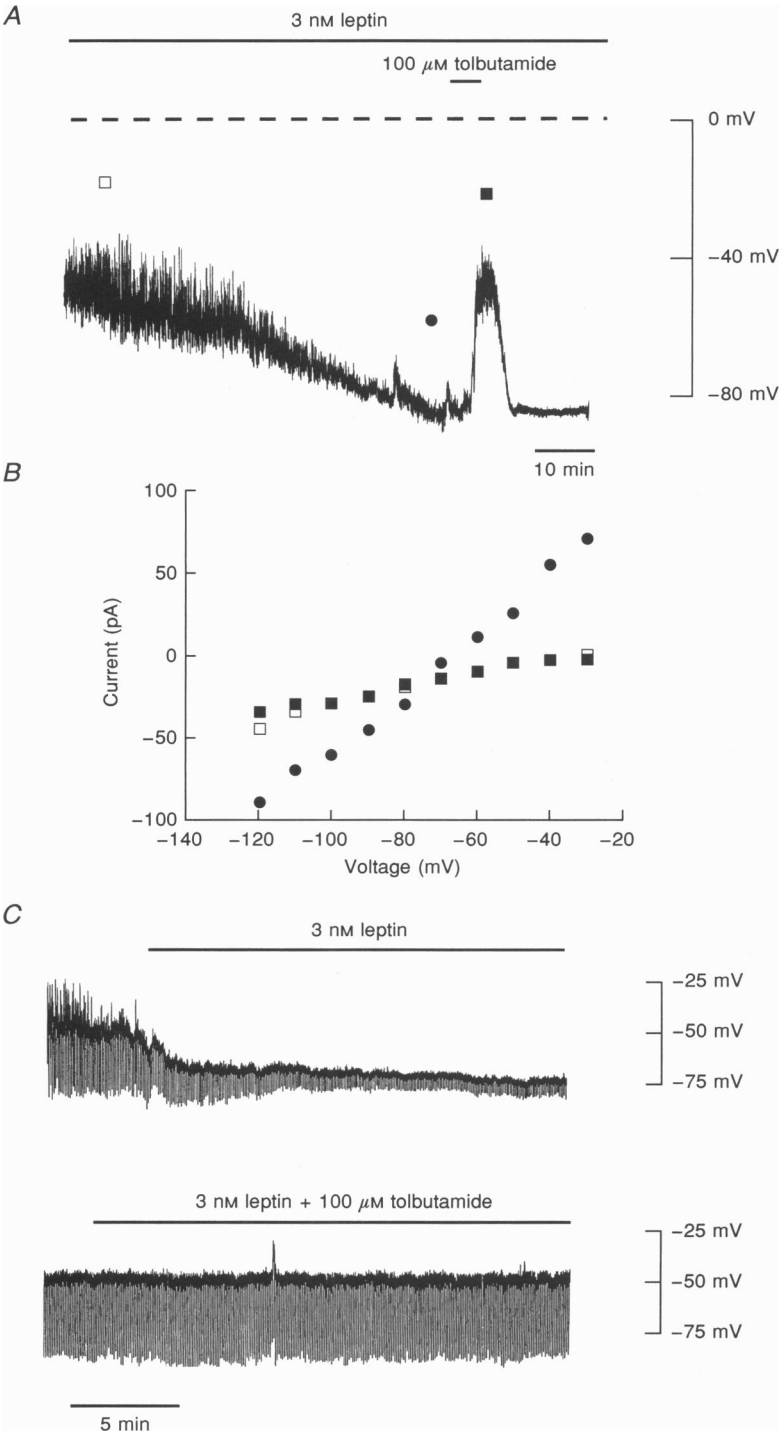

Figure 3. Leptin activates a sulphonylurea-sensitive potassium current

A, current-clamp record of membrane potential of a cell dialysed with 5 mM ATP. Leptin (3 nM) applied for the time indicated caused a membrane hyperpolarization to −80 mV, an action readily reversed by the sulphonylurea tolbutamide (100 μM). *B*, current–voltage relationships for voltage-clamped currents obtained at the points specified in *A*: control (□), leptin (●) and leptin and tolbutamide (■). Leptin increased the membrane conductance relative to control and tolbutamide reversed this leptin-induced conductance increase, with a reversal potential of −79 mV. *C*, upper and lower panels show whole-cell current clamp recordings of cells dialysed with 5 mM ATP. Application of 3 nM leptin for the time indicated by the bar (upper panel) hyperpolarized the cell from a resting membrane potential of −48 to −75 mV. This action of leptin was accompanied by an increase in conductance. In the lower panel the ability of leptin to cause membrane hyperpolarization was occluded when leptin (3 nM) was co-applied with the sulphonylurea tolbutamide (100 μM).

experiments, application of glibenclamide (0·5 μM) also reversed the actions of leptin completely, causing a recovery of the membrane potential (approximately −40 mV; $n = 8$) and conductance (reversal potential was −79 ± 2·1 mV for current blocked by leptin, indicating that it was carried by potassium ions) to pre-leptin values (data not shown). However, in contrast to the effects of tolbutamide (a first generation sulphonylurea), glibenclamide (a second generation sulphonylurea) action was not recoverable on washout. In addition, concomitant addition of leptin (3 nM) with tolbutamide (100 μM) prevented leptin from inducing a hyperpolarization or increase in potassium current ($n = 6$; Fig. 3*C*).

Identification of the K_{ATP} channel as the molecular target for acute leptin action in these cells was obtained from single-channel recordings. Application of leptin (1–10 nM)

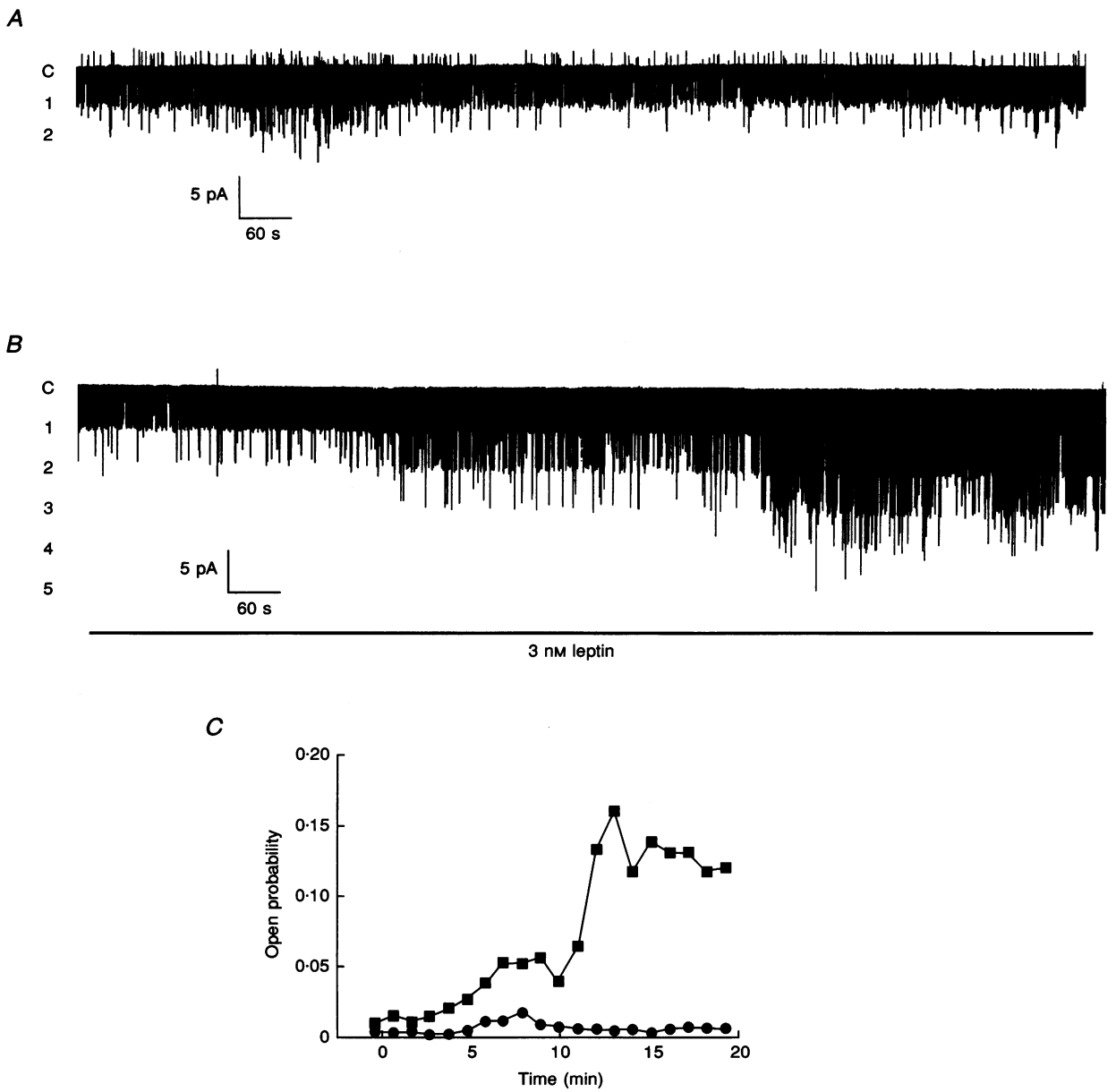

Figure 4. Activation of single potassium channel currents by leptin.

A, cell-attached recording from a CRI-G1 cell, with no applied potential and with no leptin added either to the electrode or bath solutions. Continuous recording over a 20 min period showed no significant change in channel activity with time. Upward deflections of the trace are action currents and downward deflections are channel openings. *B*, cell-attached recording from a CRI-G1 cell, at no applied potential, with 3 nM leptin present in the electrode solution only. Single-channel current activity slowly increased over the time period of recording such that initially two channels appeared to be active and after 15–20 min a maximum of five channels were active. *C*, graph of open probability against time obtained from the inside-out patches shown in *A* (●) and *B* (■).

to the bath during cell-attached recordings (with no leptin in the electrode solution) did not induce activation of K_{ATP} channel currents ($n = 5$). However, cell-attached recordings with 3 nM leptin in the electrode solution and no bath-applied leptin induced an increase in the activity of single potassium channels on ten out of thirteen occasions. There was a delay of approximately 5–20 min following formation of the cell-attached configuration before leptin-induced potassium channel activation occurred (Fig. 4*B* and *C*). Analysis of total channel current (over a 60 s period), 1 min after formation of the cell-attached configuration, resulted in a mean current of 0·59 ± 0·21 pA and this increased in the presence of 3 nM leptin, after 17–20 min to 3·40 ± 0·98 pA ($n = 6$; $P < 0·05$). Identical control experiments in the absence of leptin (e.g. Fig. 4*A* and *C*) resulted in no K_{ATP} channel activation with mean currents of 0·10 ± 0·01 and 0·11 ± 0·04 pA ($n = 3$; $P > 0·05$) at the same time periods, respectively. Once activated, channel activity continued for the duration of recordings and activity could be inhibited reversibly by the application of 100 μM tolbutamide ($n = 5$; Fig. 5) or non-reversibly by 0·5 μM glibenclamide (data not shown) to the bathing solution during the cell-attached recording configuration. Furthermore, following leptin induction of channel activity in the cell-attached recording configuration, patches were subsequently excised into the inside-out configuration, where the potassium channel activity was shown to be completely inhibited by 1 mM ATP ($n = 4$; data not shown). Thus the sulphonylurea and ATP sensitivity of the single channels activated by leptin is consistent with K_{ATP} of insulin-secreting cells (Ashcroft & Ashcroft, 1990). In contrast, application of 3 nM leptin to the extracellular aspect of outside-out membrane patches did not cause activation of K_{ATP} channel activity ($n = 11$), regardless of the concentration of pipette ATP (0·25–5·0 mM) or calcium (0·1 or 1 μM), although channel activity present in patches with lowered internal ATP displayed tolbutamide sensitivity (data not shown).

DISCUSSION

Previous studies have shown that recombinant leptin decreases the plasma insulin and glucose levels in *ob/ob* (but not lean) mice (Pelleymounter *et al.* 1995), an action unlikely to be secondary to weight loss or reduced food intake (Levin, Nelson, Gurney, Vandlen & De Sauvage, 1996). More recently, recombinant leptin has been shown to inhibit glucose-stimulated insulin secretion by isolated islets

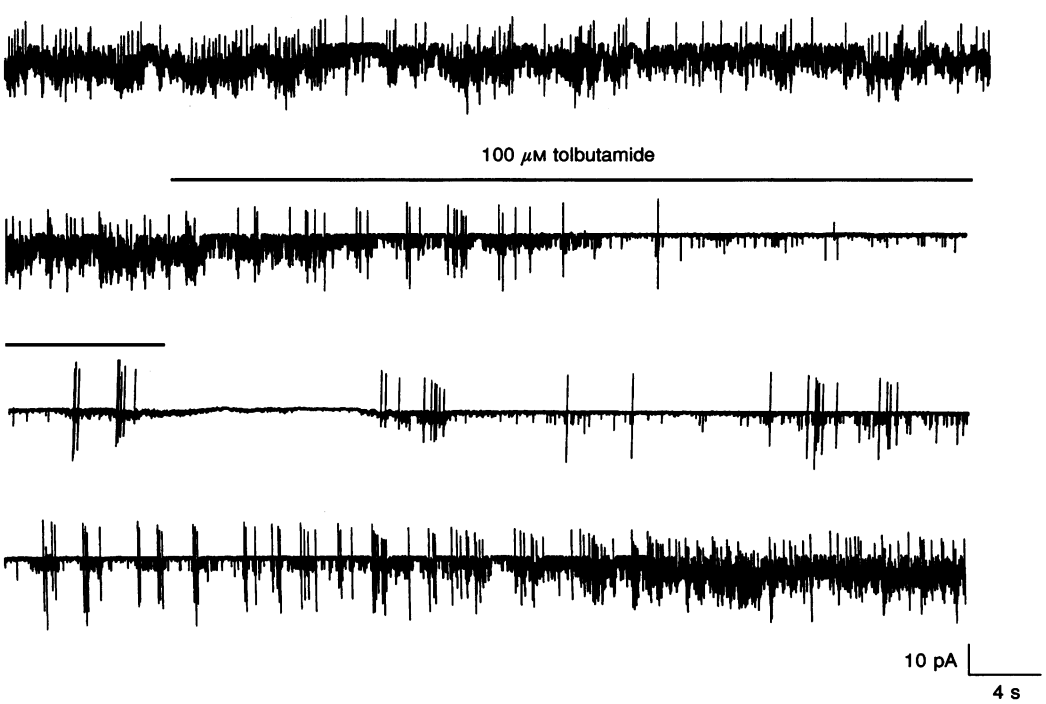

Figure 5. Effect of tolbutamide on leptin-activated single potassium channels

Representative cell-attached recording from a CRI-G1 cell with 3 nM leptin in the electrode solution and no applied voltage. One minute after formation of the cell-attached recording configuration, few K_{ATP} channels were active and the cell displayed a significant level of action potential activity (not shown). After 21 min of leptin exposure there was considerable potassium channel activity in the patch (top trace). Note that under these recording conditions the cell continued to fire action potentials due to depolarizing current entering the cell, indicating the continuation of the cell-attached recording mode. The leptin-induced channel activity was inhibited by application of 100 μM tolbutamide to the bath solution (as shown by the bar) and this effect was reversible on washout. The apparent reduction in action potential activity was as a result of the decreased potassium entry through fewer channel openings. The traces shown are continuous.

from *ob/ob* and wild-type mice (Emilsson *et al.* 1997). Our present data are consistent with leptin acting to decrease the release of insulin from pancreatic β-cells through the activation of K_{ATP} channels resulting in β-cell hyperpolarization and decreased cell excitability. The hyperglycaemic agent diazoxide (Sturgess *et al.* 1988) also acts to hyperpolarize insulin-secreting cells and decrease excitability, but its actions are reversible and more rapid in comparison with leptin. Activation of K_{ATP} channel current by diazoxide in this cell line also occludes the effect of leptin on K^+ conductance. Consequently, the identification of K_{ATP} channels as a molecular target for leptin action on CRI-G1 cells relies on three pieces of evidence: (1) that full activation of K_{ATP} currents, either by removal of intracellular ATP or by activation with the hypoglycaemic agent diazoxide, prevents the leptin-induced potassium current increase, (2) the sulphonylureas tolbutamide and glibenclamide (purported selective K_{ATP} channel inhibitors; Ashcroft & Ashcroft, 1992) reverse or prevent leptin-induced potassium current activation and cell hyperpolarization; and (3) identification of the channel activated by leptin as a sulphonylurea-sensitive ATP-inhibited K^+ channel by single-channel recordings.

Leptin opened K_{ATP} channels during cell-attached recordings only when the protein was present in the electrode solution and therefore in direct contact with the extracellular domain of the membrane patch. This result suggests that leptin, presumably following binding to a high-affinity receptor site (Kieffer *et al.* 1996), probably opens K_{ATP} channels in a membrane-delimited manner, although formation of the outside-out configuration ablates this effect. Leptin receptors belong to the class I cytokine receptor family (Tartaglia *et al.* 1995) and recently another member of this family, the prolactin receptor, was demonstrated to couple to large conductance calcium-activated K^+ channels stably transfected into Chinese hamster ovary cells (Prevarskaya, Skryma, Vacher, Daniel, Djiane & Dufy, 1995) in a membrane-delimited manner. This class of cytokine receptor is considered to act through the Janus protein tyrosine kinase (JAK) and signal transducers and activators of transcription (STAT) signal transduction pathways, and there is evidence that leptin receptor activation does use these pathways. However, further experiments are required to determine whether leptin receptor coupling to the K_{ATP} channel in insulin-secreting cells is via tyrosine kinase phosphorylation, as reported for the prolactin receptor (Prevarskaya *et al.* 1995). The concentrations of leptin shown to activate K_{ATP} channels in the present study are generally less than that reported for significant inhibition of insulin release (Emilsson *et al.* 1997), but certainly within the range reported in human plasma (0·3–3 nM; Caro, Sinha, Kolaczynski, Zhang & Considine, 1996).

There is now good evidence to suppose that there is a feedback loop between adipose tissue and pancreatic β-cells. Administration of insulin to mice increases leptin expression in adipose tissue and administration of leptin clearly reduces plasma insulin levels. There is also a positive correlation between the mass of adipose tissue and leptin levels (Caro *et al.* 1996). Therefore increased adipose mass will result in raised plasma leptin levels and this in turn will feed back to pancreatic β-cells to inhibit insulin secretion, via stimulation of K_{ATP} channels, and so result in reduced anabolism. However in the obese state this feedback loop is considered to be dysfunctional. In diet-induced obese animals, higher doses of leptin are required to induce weight loss (Campfield *et al.* 1995) and studies in humans have indicated that the majority of human obese subjects are hyperleptinaemic (Sinha *et al.* 1996). Furthermore, there is no evidence for a defect in leptin production or in the leptin receptor in humans (Considine *et al.* 1996; Maffei *et al.* 1996). Consequently, leptin resistance at a site downstream from the receptor may be an important factor in the aetiology of human obesity and diabetes mellitus. Hence further studies on leptin receptor K_{ATP} channel transduction mechanisms may help to elucidate the nature of these defects.

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