

Role of tyrosine phosphorylation in leptin activation of ATP-sensitive K⁺ channels in the rat insulinoma cell line CRI-G1

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1. Using whole-cell and cell-attached recording configurations, the role of phosphorylation in leptin activation of ATP-sensitive K⁺ (K_{ATP}) channels was examined in the rat CRI-G1 insulinoma cell line.
2. Whole-cell current clamp recordings demonstrated that, following dialysis with the non-hydrolysable ATP analogue 5'-adenylylimidodiphosphate (AMP-PNP; 3–5 mM), the leptin-induced hyperpolarization and increase in K⁺ conductance were completely inhibited.
3. Under current clamp conditions, application of the broad-spectrum protein kinase inhibitor H-7 (10 μM) had no effect on the resting membrane potential or slope conductance of CRI-G1 insulinoma cells and did not occlude the actions of leptin.
4. Application of the tyrosine kinase inhibitors genistein (10 μM), tyrphostin B42 (10 μM) and herbimycin A (500 nM) all resulted in activation of K_{ATP} channels. In cell-attached recordings, the presence of tyrphostin B42 (10 μM) in the pipette solution activated tolbutamide-sensitive K_{ATP} channels in CRI-G1 cells. In contrast, the inactive analogues of genistein and tyrphostin B42 were without effect.
5. The serine/threonine-specific protein phosphatase inhibitors okadaic acid (50 nM) and cyclosporin A (1 μM) did not prevent or reverse leptin activation of K_{ATP} channels. In contrast, whole-cell dialysis with the tyrosine phosphatase inhibitor orthovanadate (500 μM) prevented the actions of both leptin and tyrphostin B42.
6. In conclusion, leptin activation of K_{ATP} channels appears to require inhibition of tyrosine kinases and subsequent dephosphorylation. This process is likely to occur prior to activation of phosphoinositide 3-kinase (PI 3-kinase) as wortmannin prevented activation of K_{ATP} channels by tyrphostin B42.

It is well established that protein tyrosine kinases regulate a variety of cellular functions including proliferation, differentiation and signalling processes. Although a number of distinct tyrosine kinases and phosphatases have been identified (Levitzi & Gazit, 1995), the physiological actions and the intracellular targets of these proteins remain unclear. There is, however, increasing evidence that tyrosine kinases and phosphatases can modulate a variety of ion channels by either increasing or decreasing channel activity (Siegelbaum, 1994).

In pancreatic β-cells (Keiffer, Heller, Leech, Holz & Habener, 1997) and the CRI-G1 insulin-secreting cell line (Harvey, McKenna, Herson, Spanswick & Ashford, 1997), leptin, the *ob* gene product, activates ATP-sensitive potassium (K_{ATP}) channels, an action consistent with suppression of insulin

secretion. The leptin receptor shows sequence homology with members of the class I cytokine receptor superfamily (Tartaglia *et al.* 1995), which are thought to signal via association with tyrosine kinases of the janus kinase (JAK) family. Indeed, the long form of the leptin receptor (OB-Rb) activates JAK2 in a haematopoietic cell line (Ghilardi & Skoda, 1997). Several pathways can be activated by JAKs including the insulin receptor substrate proteins (Ihle, 1995). Phosphoinositide 3-kinase (PI 3-kinase) is just one of many proteins associated with the signalling downstream of insulin receptor substrate-1 (IRS-1; Myers & White, 1996). Recently, we have shown that the ability of leptin to activate K_{ATP} channels is not only regulated by insulin but also that the pathway underlying this action of leptin involves activation of PI 3-kinase (J. Harvey & M. L. J. Ashford, unpublished observations). Prolactin is also capable

of activating JAK2 (Prevarskaya, Skryma, Vacher, Daniel, Djiane & Dufy, 1995) and PI 3-kinase (Berlanga, Gualillo, Buteau, Applanat, Kelly & Edery, 1997), thus the signalling capabilities of the leptin receptor in CRI-G1 cells may show parallels to those of other class I cytokine receptors. Since tyrosine phosphorylation plays a critical role in the actions of other cytokines, we have examined the effects of inhibitors of tyrosine kinases and phosphatases in the present study, in order to elucidate further the mechanism underlying leptin activation of K_{ATP} channels in CRI-G1 insulinoma cells.

In addition to protein tyrosine kinases, the activity of ion channels can be modulated by serine/threonine-specific protein kinases (Jonas & Kaczmarek, 1996). Indeed phorbol ester-induced activation of protein kinase C results in phosphorylation and subsequent activation of K_{ATP} channels in insulin-secreting cells (Ribalet, Eddlestone & Ciani, 1988; De Weille, Schmid-Antomarchi, Fosset & Lazdunski, 1989). Furthermore, in another insulin-secreting cell line RINm5F (Ribalet, Ciani & Eddlestone, 1989) and rabbit arterial smooth muscle (Quayle, Bonev, Brayden & Nelson, 1994), K_{ATP} channel activity is enhanced via protein kinase A-dependent phosphorylation. Consequently we have also examined whether leptin activates K_{ATP} channels in CRI-G1 cells via serine/threonine-specific protein kinases. We have reported previously that tyrosine kinase inhibitors mimic leptin activation of K_{ATP} channels in CRI-G1 insulin-secreting cells (Ashford & Harvey, 1997).

METHODS

Cell culture

Cells from the rat insulin-secreting cell line CRI-G1 were grown in Dulbecco's modified Eagle's medium with sodium pyruvate and glucose (Life Technologies), supplemented with 10% fetal calf serum (Sigma) and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Cells were passaged every 2–5 days as described previously (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 clamp recording to monitor membrane potential with excursions to voltage clamp mode to examine macroscopic currents and the cell-attached configuration to examine single channel responses, as described previously (Harvey *et al.* 1997). During voltage clamp recordings, the membrane potential was clamped at –50 mV and 10 mV steps of 100 ms duration were applied every 200 ms (range, –120 to –30 mV). Current and voltage were measured using the Axoclamp 200B amplifier (Axon Instruments) for whole-cell recordings and a List EPC-7 for cell-attached single channel recordings. Currents evoked in response to the voltage step protocol were analysed using pCLAMP 6.0 software (Axon Instruments) whereas current clamp data were recorded onto digital audiotapes and replayed for illustration on a Gould TA 240 chart recorder. Single channel data were analysed for current amplitude and channel activity ($N_f P_o$; where N_f is the number of functional channels and P_o is the open probability) as described previously (Lee, Rowe & Ashford, 1995). All data are expressed as

means \pm s.e.m. and statistical analyses were performed using Student's unpaired *t* test (unless otherwise stated). $P < 0.05$ was considered significant.

Recording electrodes were pulled from borosilicate glass and had resistances of 1–5 M Ω for whole-cell recordings and 8–12 M Ω for cell-attached experiments when filled with electrolyte solution. The pipette solution for whole-cell recordings comprised (mM): 140 KCl, 0.6 MgCl₂, 2.73 CaCl₂, 5.0 ATP, 10 EGTA, 10 Hepes, pH 7.2 (free [Ca²⁺] of 100 nM), whereas for cell-attached single channel recordings the pipette solution contained (mM): 140 KCl, 1 CaCl₂, 1 MgCl₂, 10 Hepes, pH 7.2. The bath solution was normal saline (mM): 135 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 Hepes, pH 7.4. 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).

Tyrphostin B42, genistein, daidzein, tyrphostin 1, ATP, 5'-adenylylimidodiphosphate (AMP-PNP), tolbutamide, sodium orthovanadate, herbimycin A and cyclosporin A were all obtained from Sigma. Recombinant human leptin was supplied by Dr Peter Lind of Pharmacia-Upjohn (Stockholm, Sweden). Okadaic acid was obtained from Research Biochemicals International. Tolbutamide was made up as a 100 mM stock solution in DMSO, whereas ATP and AMP-PNP were stored as 100 mM stock solutions in 10 mM Hepes (pH 7.2) and kept at –4 °C until required. Genistein, tyrphostin B42, daidzein, herbimycin A and tyrphostin 1 were all made up as 10 mM stock solutions in DMSO. Okadaic acid was made up as a 50 μ M stock in DMSO and cyclosporin A was stored as a 1 mM stock in 100% ethanol.

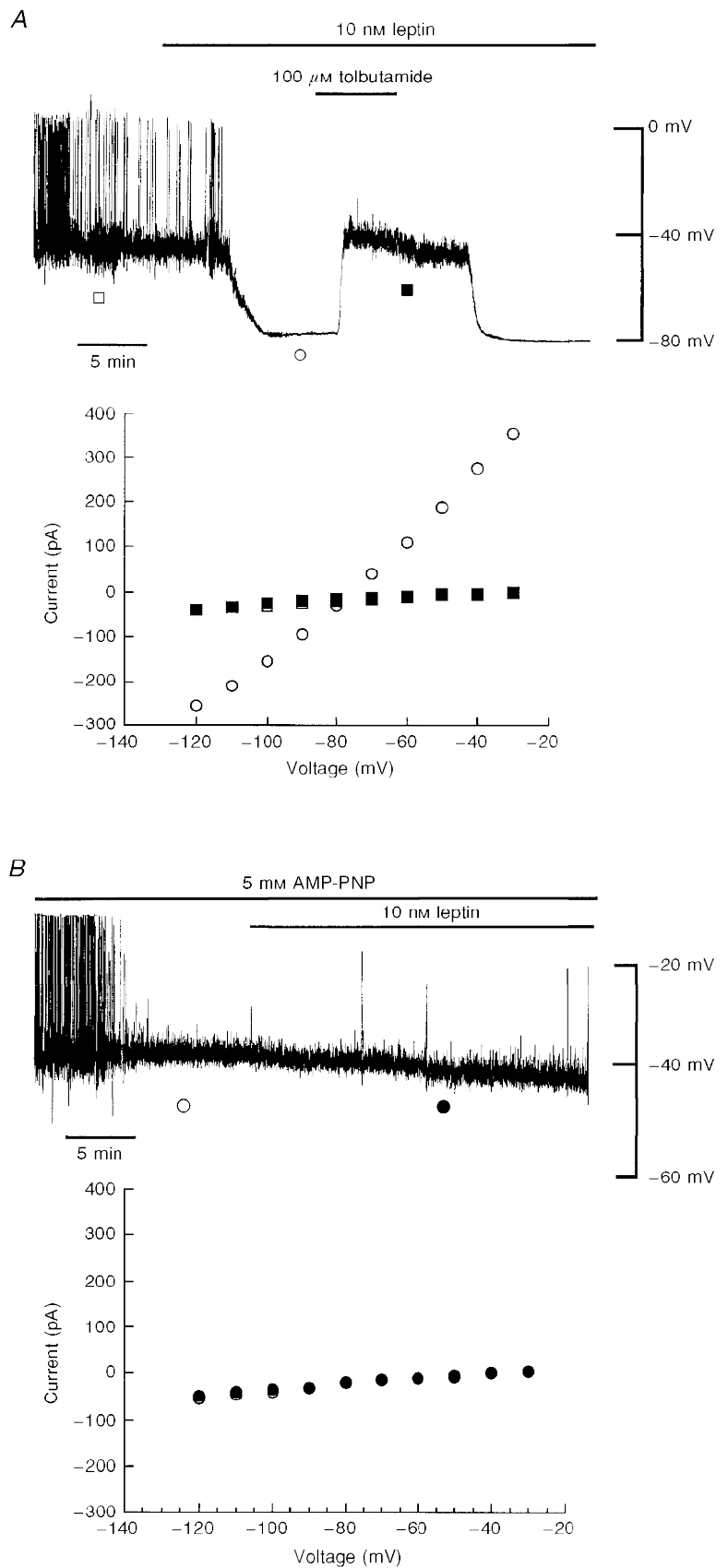
RESULTS

Leptin activates K_{ATP} channels

Under current clamp conditions with 5 mM ATP present in the electrode solution to maintain K_{ATP} channels in the closed state, the mean resting membrane potential of CRI-G1 cells was -38 ± 1.7 mV ($n = 68$). Application of the *ob* gene product leptin (10 nM) hyperpolarized the CRI-G1 cells to -73 ± 3.1 mV ($n = 5$; Fig. 1A). Examination of the voltage clamped macroscopic currents indicated that prior to the addition of leptin the slope conductance of the cells was 0.57 ± 0.06 nS and that following exposure to leptin there was an increase in the slope conductance to 7.84 ± 1.23 nS ($n = 5$; Fig. 1A). The reversal potential (obtained from the point of intersection of the current–voltage relationships) associated with the leptin increase in conductance was -79 ± 1.8 mV ($n = 5$), which is close to calculated value for the K⁺ equilibrium potential (E_K ; –84 mV) in this system, indicating an increase in K⁺ conductance. The sulphonylurea tolbutamide (100 μ M) completely reversed the leptin-induced hyperpolarization and increase in conductance to pre-leptin levels, such that the membrane potential and slope conductance values in the presence of tolbutamide were -41 ± 2.1 mV and 0.61 ± 0.05 nS, respectively ($n = 4$). Thus these data indicate that leptin activates K_{ATP} channels in CRI-G1 insulin-secreting cells, in agreement with previous studies using this cell line (Harvey *et al.* 1997) and pancreatic β -cells (Keiffer *et al.* 1997).

Figure 1. Leptin activation of K_{ATP} channels is blocked by AMP-PNP

A, top panel: whole-cell current clamp record of a CRI-G1 cell following dialysis with a 5 mM ATP-containing electrode solution. In this and subsequent figures, the trace begins approximately 5 min after obtaining the whole-cell configuration. Application of leptin (10 nM) for the time indicated resulted in hyperpolarization of the membrane from -39 to -80 mV, an action readily reversed by the sulphonylurea tolbutamide ($100 \mu\text{M}$). Note that there is a delay of around 2 min from the time of drug addition until complete bath exchange. Bottom panel: plot of the current–voltage relationships for voltage clamped currents obtained at the points specified in the top panel: \square , control; \circ , leptin; \blacksquare , leptin and tolbutamide. Leptin increased the membrane conductance relative to control and tolbutamide ($100 \mu\text{M}$) reversed the leptin-induced increase in conductance with a reversal potential of -79 mV. *B*, top panel: current clamp record of a cell dialysed with 5 mM AMP-PNP. Application of leptin (10 nM) for the time indicated, after at least 15–20 min dialysis with AMP-PNP, failed to hyperpolarize CRI-G1 cells. Bottom panel: plot of the current–voltage relationships for the voltage clamped currents in the top panel. Leptin (\bullet) had no effect on the membrane conductance relative to control (\circ). AMP-PNP prevented leptin activation of K_{ATP} channels.



Leptin activation of K_{ATP} channels is prevented by AMP-PNP

In order to investigate whether the mechanism underlying leptin activation of K_{ATP} channels involves a phosphorylation-dependent process, the effects of AMP-PNP, a non-hydrolysable analogue of ATP, which does not serve as a substrate for protein kinases, were examined. In this series of experiments 5 mM ATP was replaced with AMP-PNP (3–5 mM) in the pipette solution. In current clamp mode under these conditions, the mean resting membrane potential and slope conductance values, 10–15 min after obtaining the whole-cell configuration, were -39 ± 1.7 mV and 0.48 ± 0.05 nS, respectively ($n = 7$; Fig. 1B). After at least 20 min of recording (to allow complete dialysis of AMP-PNP into the cell interior), leptin (10 nM) was applied to the bath. In contrast to its actions under control conditions (following dialysis with an electrode solution containing 5 mM ATP), leptin had no effect on CRI-G1 insulinoma cells. Thus, the membrane potential and slope conductance values obtained after 15–20 min exposure to leptin were -38 ± 1.9 mV and 0.52 ± 0.06 nS, respectively ($n = 7$). These data are consistent with leptin activation of K_{ATP} channel currents involving a phosphorylation-dependent process.

H-7 does not occlude leptin activation of K_{ATP} channels

Since protein kinases are capable of phosphorylating serine/threonine residues of a range of proteins we examined their role in the actions of leptin using the broad-spectrum protein kinase inhibitor H-7 (Hidaka, Watanabe & Kobayashi, 1991). At the concentration (10 μ M) examined, H-7 inhibits protein kinases A, C and G (Hidaka *et al.* 1991). Under current clamp conditions with 5 mM ATP in the pipette solution, application of H-7 (10 μ M) for 10–15 min had no effect on the resting membrane potential of CRI-G1 cells; the mean resting membrane potential in the presence of H-7 (10 μ M) was -33 ± 2.0 mV ($n = 4$; Fig. 2A). Examination of the macroscopic currents under voltage clamp conditions indicated that H-7 had no effect on the slope conductance of CRI-G1 cells as the slope conductance values obtained in the absence and presence of H-7 (10 μ M) were 0.53 ± 0.07 and 0.55 ± 0.06 nS, respectively ($n = 4$). Coapplication of leptin (10 nM) and H-7 (10 μ M), after 10–15 min exposure to H-7, resulted in hyperpolarization of cells to -68 ± 4.2 mV ($n = 4$; Fig. 2B). The leptin-induced hyperpolarization was accompanied by an increase in slope conductance to 2.56 ± 0.34 nS and the reversal potential associated with this increase was -79 ± 0.86 mV ($n = 4$). In parallel control experiments performed in the absence of H-7, application of leptin (10 nM) hyperpolarized CRI-G1 cells to -70 ± 3.8 mV ($n = 4$) with a concomitant increase in slope conductance from 0.54 ± 0.07 to 2.81 ± 0.26 nS ($n = 4$). Tolbutamide (100 μ M) completely reversed the actions of leptin after exposure to H-7 with a reversal potential of -80 ± 0.94 mV ($n = 3$). Thus blockade of serine- or threonine-specific

protein kinases does not occlude leptin activation of K_{ATP} channels in CRI-G1 insulinoma cells.

Tyrosine kinase inhibitors mimic the actions of leptin

In order to determine whether tyrosine phosphorylation is involved in the actions of leptin, the effects of three different tyrosine kinase inhibitors were investigated. Genistein and tyrphostin B42 (Levitzki & Gazit, 1995) are both broad-spectrum tyrosine kinase inhibitors that act via reversible competitive inhibition of ATP and non-competitive inhibition of the protein substrate. In contrast, herbimycin A (Levitzki & Gazit, 1995) inhibits a broad range of tyrosine kinases via competitive inhibition of the protein substrate in an irreversible manner. Under current clamp conditions with 5 mM ATP in the electrode solution to maintain K_{ATP} channels in the closed state, application of tyrphostin B42 (10 μ M), genistein (10 μ M) or herbimycin A (500 nM) resulted in hyperpolarization of the membrane from -39 ± 1.0 mV ($n = 24$) to -73 ± 1.7 mV ($n = 10$), -58 ± 3.1 mV ($n = 8$) and -71 ± 2.6 mV ($n = 6$), respectively (Fig. 3B and D). Under voltage clamp conditions examination of the macroscopic currents indicated that the hyperpolarization induced by these agents was accompanied by an increase in the slope conductance from 0.53 ± 0.06 to 5.27 ± 0.92 nS ($n = 10$, tyrphostin B42; Fig. 3D), from 0.56 ± 0.06 to 2.4 ± 0.68 nS ($n = 8$, genistein; Fig. 3B) and from 0.64 ± 0.06 to 6.23 ± 1.63 nS ($n = 6$, herbimycin A; data not shown). The increase in slope conductance induced by genistein was significantly less ($P < 0.05$) than the K^+ conductance increase induced by either tyrphostin B42 or herbimycin A. The reversal potentials associated with this increase in conductance were -79 ± 0.9 mV ($n = 10$; tyrphostin B42), -78 ± 0.8 mV ($n = 8$; genistein) and -78 ± 0.71 mV ($n = 6$; herbimycin A), consistent with an increase in K^+ conductance. In contrast, the inactive tyrphostin analogue tyrphostin 1 (Levitzki & Gazit, 1995) had no effect on CRI-G1 cells, such that the membrane potential and slope conductance values obtained after 15–20 min exposure to tyrphostin 1 (10 μ M) were -32 ± 2.4 mV and 0.56 ± 0.09 nS, respectively ($n = 3$; Fig. 3C). Furthermore, application of daidzein (10 μ M), the inactive analogue of genistein (Levitzki & Gazit, 1995), for at least 20 min also failed to affect either the membrane potential or slope conductance of these cells, with values of -39 ± 1.2 mV and 0.68 ± 0.03 nS, respectively ($n = 3$; Fig. 3A).

The sulphonylurea tolbutamide (100 μ M) completely reversed the hyperpolarization and increase in slope conductance induced by all three tyrosine kinase inhibitors to pre-drug levels. The reversal potentials associated with this action of tolbutamide were -79 ± 1.1 mV ($n = 6$, tyrphostin B42; Fig. 3D), -78 ± 1.4 mV ($n = 4$, genistein; Fig. 3B) and -78 ± 0.81 mV ($n = 6$, herbimycin A; data not shown). Together these data indicate that inhibition of tyrosine kinase activity results in activation of K_{ATP} channels in CRI-G1 insulin-secreting cells. Cell-attached recordings support these observations and clearly demonstrate that inhibition of tyrosine kinases activates

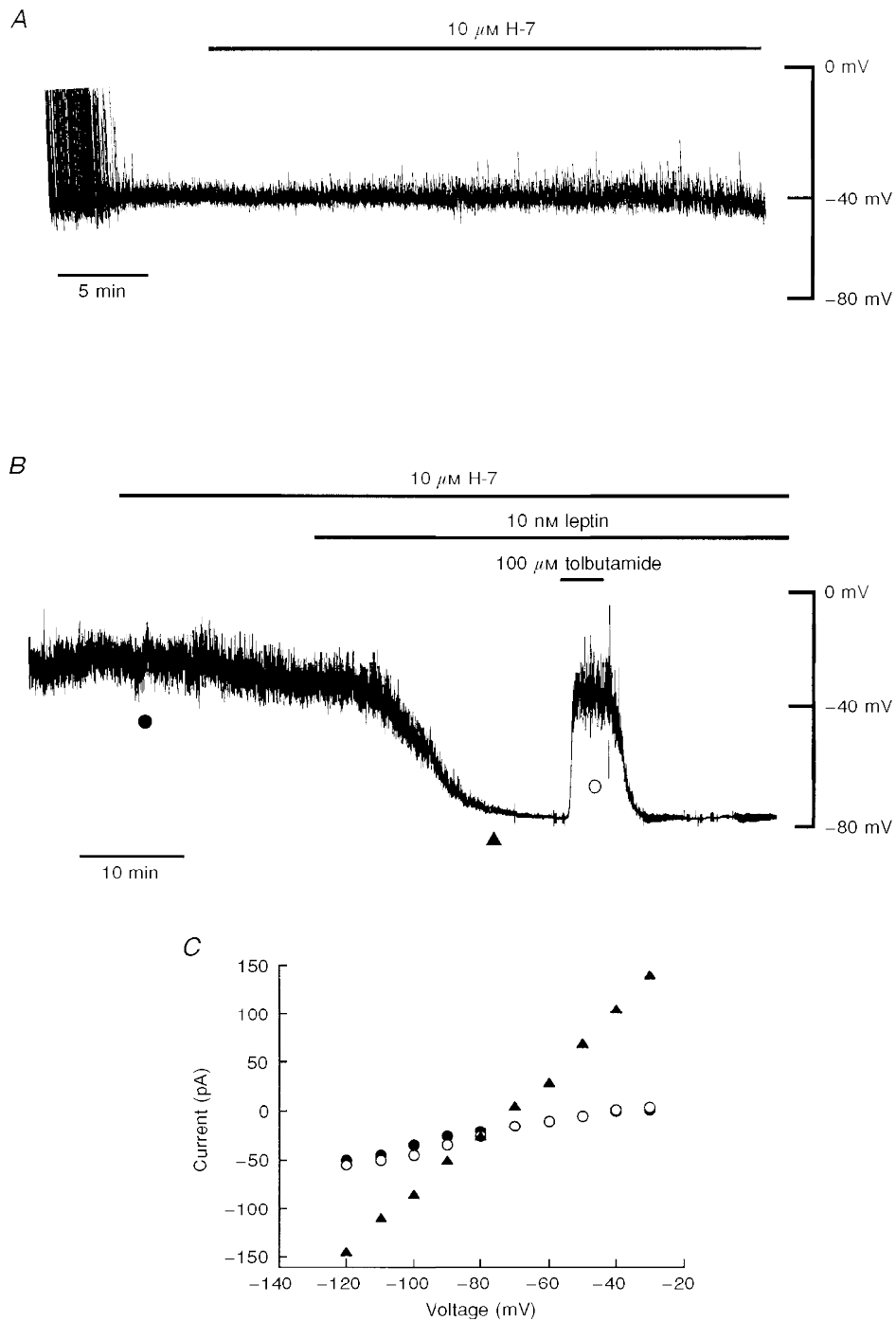


Figure 2. H-7 does not prevent leptin activation of K_{ATP} channels

A, current clamp record of a cell dialysed with an electrode solution containing 5 mM ATP. Addition of H-7 (10 μM) for approximately 20 min (as indicated by the bar) had no effect on the resting membrane potential of CRI-G1 cells. *B*, current clamp record of a cell dialysed with 5 mM ATP-containing electrode solution. Application of H-7 (10 μM) for the time indicated had no effect on the resting membrane potential. Addition of leptin (10 nM) 10–15 min after prior exposure to H-7 (10 μM) resulted in hyperpolarization of CRI-G1 cells from -40 to -78 mV. Tolbutamide (100 μM) applied after the leptin-induced hyperpolarization caused complete reversal of the membrane potential to pre-leptin levels. *C*, plot of the current–voltage relationships for the voltage clamped currents obtained at the points specified in *B*: ●, H-7; ▲, leptin and H-7; ○, tolbutamide. H-7 (10 μM) did not prevent the leptin-induced increase in K^+ conductance.

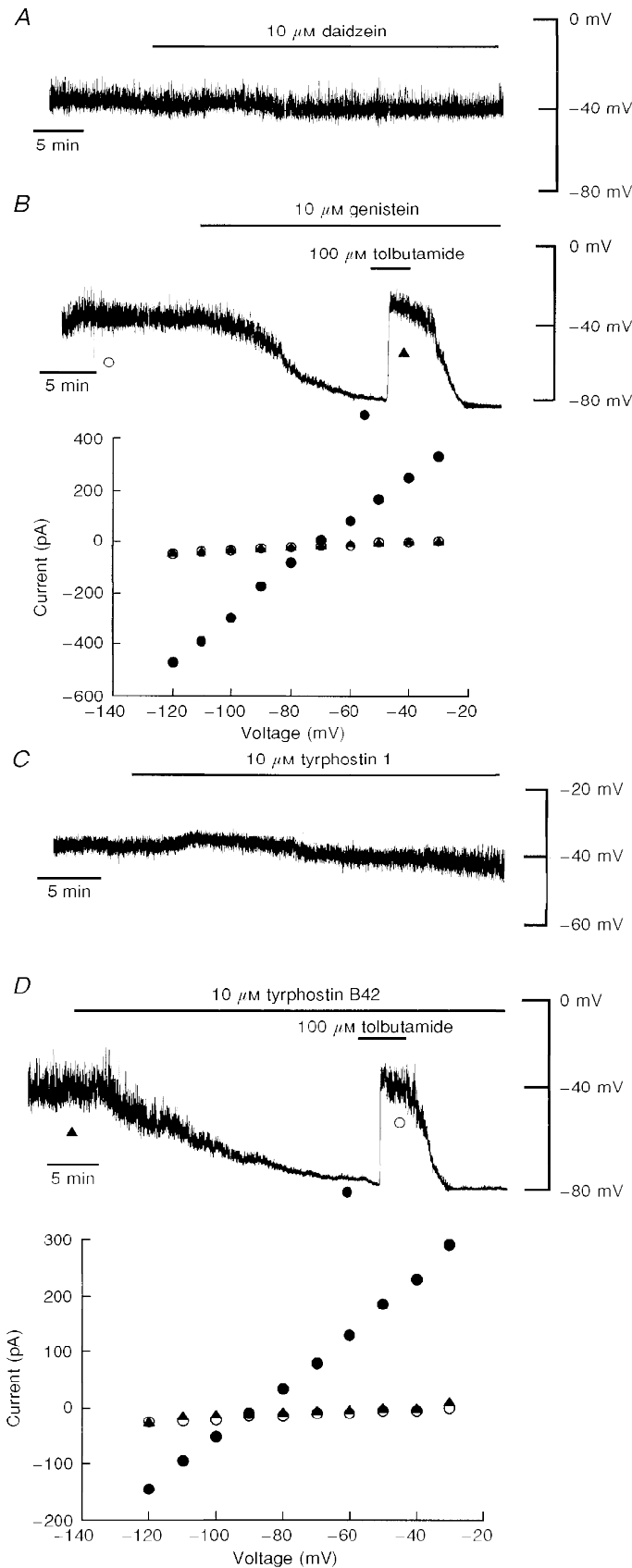


Figure 3. Tyrosine kinase inhibitors activate K_{ATP} channel currents

A, current clamp record of a cell dialysed with an electrode solution containing 5 mM ATP. Application of daidzein (10 μM) had no effect on the resting membrane potential of the cell. *B*, top panel: current clamp trace of a cell dialysed with 5 mM ATP. Addition of genistein (10 μM) resulted in hyperpolarization of the cell membrane to -80 mV. The sulphonylurea tolbutamide (100 μM) completely reversed the genistein-induced hyperpolarization to pre-genistein levels. Bottom panel: plot of the current-voltage relationships for the voltage clamped currents shown at the top: \circ , control; \bullet , genistein; \blacktriangle , genistein and tolbutamide. The genistein-induced hyperpolarization was accompanied by an increase in K^+ conductance. Tolbutamide readily reversed this action of genistein with a reversal potential of -77 mV. *C*, current clamp record of a CRI-G1 cell dialysed with 5 mM ATP. Application of tyrphostin 1 (10 μM) for at least 25 min failed to affect the resting membrane potential of the cell. *D*, top panel: current clamp record of a cell dialysed with 5 mM ATP. Application of tyrphostin B42 (10 μM) resulted in hyperpolarization of the CRI-G1 cell to -78 mV. Tolbutamide (100 μM) completely reversed the tyrphostin-induced hyperpolarization to pre-tyrphostin levels. Bottom panel, plot of the current-voltage relationships for the points specified in the top panel: \blacktriangle , control; \bullet , tyrphostin B42; \circ , tyrphostin B42 and tolbutamide. Tyrphostin B42 increased the membrane conductance relative to control and tolbutamide reversed this action of tyrphostin with a reversal potential of -83 mV.

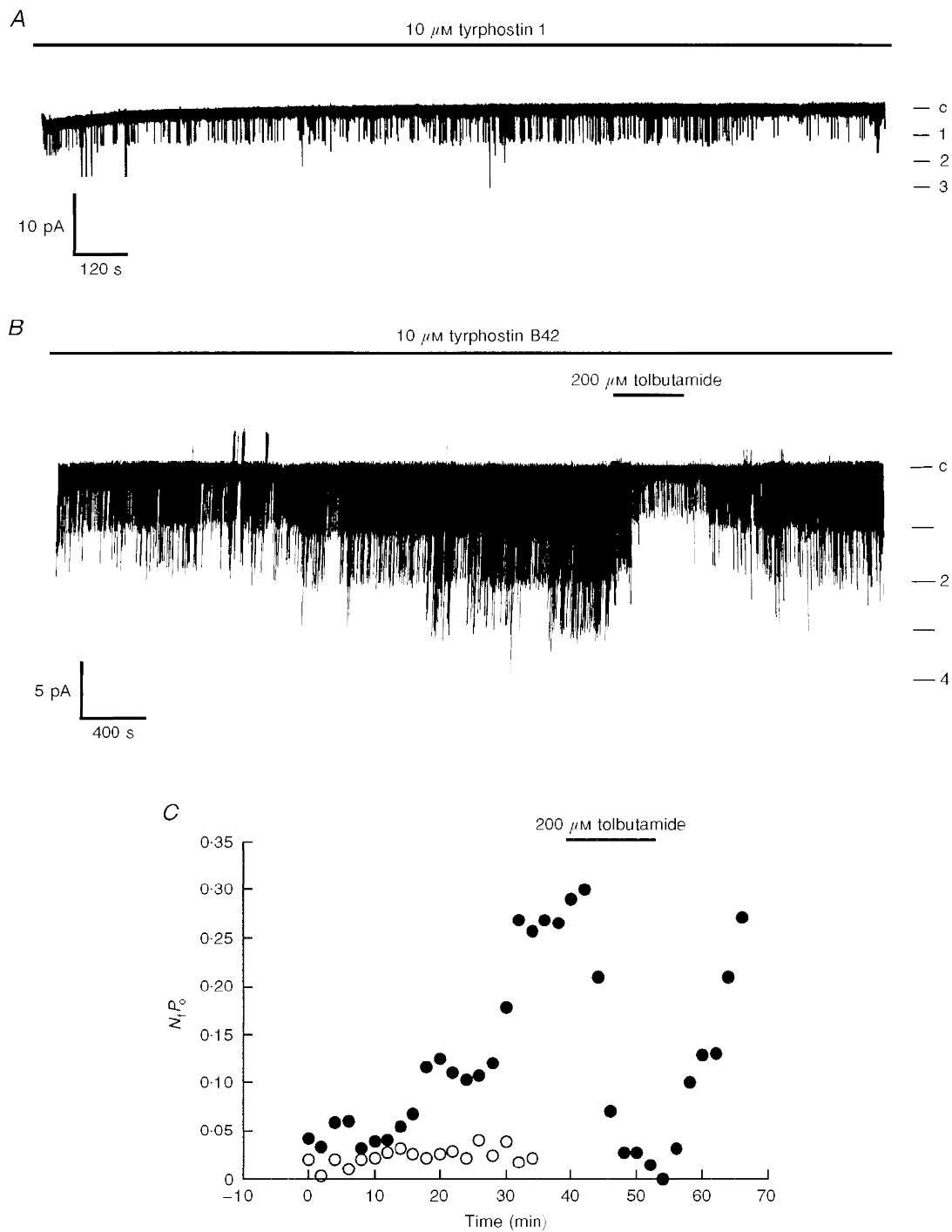


Figure 4. Activation of single K⁺ channel currents by tyrosine kinase inhibitors

A, cell-attached recording from a CRI-G1 cell, at 10 mV applied to the recording pipette and with 10 μM tyrphostin 1 present in the electrode solution. Continuous recording over a 30 min period showed no significant change in channel activity with time. Note that under these recording conditions channel openings are denoted as downward deflections (inward currents). *B*, cell-attached recording of a CRI-G1 cell at 10 mV applied potential and with 10 μM tyrphostin B42 in the electrode solution. Single channel 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 four channels were active. In *A* and *B*, c indicates the closed channel level. *C*, graph of channel activity ($N_r P_o$) against time obtained from the cell-attached patches shown in *A* (○) and *B* (●).

K_{ATP} channels (Fig. 4). Application of tyrphostin B42 ($10 \mu\text{M}$) to the bath during cell-attached recordings (with no tyrphostin B42 in the electrode) did not induce activation of K_{ATP} channel currents ($n = 7$). However, recordings with $10 \mu\text{M}$ tyrphostin B42 in the electrode solution resulted in activation of K_{ATP} channels within 25 min ($n = 6$) and channel activity was inhibited by application of the sulphonylurea tolbutamide ($200 \mu\text{M}$; $n = 4$; Fig. 4B). Analysis of total channel current (over a 120 s period) 2–4 min after formation of the cell-attached configuration resulted in a mean current of 0.26 ± 0.07 pA and this increased to 2.41 ± 0.49 pA after 20–22 min ($n = 5$; $P < 0.05$). In contrast, cell-attached recordings with the inactive analogue of tyrphostin B42, tyrphostin 1 ($10 \mu\text{M}$), in the pipette solution for at least 25 min failed to activate K_{ATP} channels ($n = 4$; Fig. 4A). The mean currents obtained at the same time periods were 0.13 ± 0.04 and 0.14 ± 0.05 pA, respectively ($n = 4$; $P > 0.05$).

Application of leptin (10 nM) following the tyrphostin B42-induced hyperpolarization and increase in conductance had no significant effect on the membrane potential or slope conductance (Fig. 5C and D). The mean cell membrane potential was -76 ± 2.3 and -78 ± 2.9 mV ($n = 5$; $P > 0.05$) and the slope conductance was 3.9 ± 0.6 and 4.2 ± 0.5 nS ($n = 5$; $P > 0.05$) in the absence and presence of leptin, respectively. However, in the presence of genistein ($10 \mu\text{M}$), which itself hyperpolarized CRI-G1 cells to -52 ± 3.5 mV, leptin (3–10 nM) further hyperpolarized the cells to -80 ± 2.5 mV ($n = 4$; $P > 0.05$). This action of leptin was associated with a further increase in conductance from 1.5 ± 0.4 to 6.5 ± 3.4 nS ($n = 4$) and was completely reversed by tolbutamide to pre-genistein levels ($100 \mu\text{M}$; $n = 4$; Fig. 5A). These data indicate that leptin activation of K_{ATP} channels is mimicked by three different tyrosine kinase inhibitors and that activation of K_{ATP} channels may require dephosphorylation of some unidentified component of the pathway.

Serine/threonine-specific phosphatase inhibitors failed to prevent or reverse leptin activation of K_{ATP} channels

Since inhibition of tyrosine phosphorylation appears to be required for activation of K_{ATP} channels, the next series of experiments investigated whether the effects of leptin could be prevented or reversed by inhibition of dephosphorylation using protein phosphatase inhibitors. Two different serine/threonine-specific protein phosphatase inhibitors were examined, which show specificity for distinct phosphatases; okadaic acid is a potent inhibitor of protein phosphatases 1 and 2A (PP1 and PP2A; Haystead *et al.* 1989) whereas cyclosporin A is a membrane-impermeant inhibitor of protein phosphatase 2B (PP2B; Liu, Farmer, Lane, Friedman, Weissman & Schreiber, 1991).

In order to determine whether inhibition of PP1 and PP2A prevents leptin activation of K_{ATP} channels, cells were exposed to okadaic acid prior to the addition of leptin. In

four cells dialysed with an electrode solution containing 5 mM ATP, application of okadaic acid (10–50 nM) for 15–20 min had no effect on the resting membrane potential (-38 ± 2.1 mV; $n = 4$) and slope conductance (0.38 ± 0.06 nS; $n = 4$; Fig. 6A). Addition of leptin (10 nM) after 20–30 min exposure to okadaic acid resulted in hyperpolarization of CRI-G1 cells to -70 ± 2.3 mV with an associated increase in slope conductance to 8.63 ± 1.1 nS ($n = 4$; Fig. 6A). This action of leptin was due to activation of K_{ATP} channels as the sulphonylurea tolbutamide ($100 \mu\text{M}$) completely reversed the actions of leptin with a reversal potential of -77 ± 1.8 mV ($n = 4$; Fig. 6A). Thus these data suggest that the ability of leptin to activate K_{ATP} channels is not prevented by inhibition of PP1 and PP2A.

Similarly, bath application of okadaic acid (50 nM) following the addition of leptin was unable to reverse leptin activation of K_{ATP} channels. In cells dialysed with 5 mM ATP, application of leptin (10 nM) hyperpolarized CRI-G1 cells from -41 ± 1.9 to -71 ± 4.8 mV (Fig. 6B) with a concomitant increase in slope conductance from 0.47 ± 0.06 to 6.91 ± 1.6 nS ($n = 5$). However, following activation of K_{ATP} channels by leptin (10 nM), coapplication of okadaic acid (50 nM) and leptin (10 nM) for at least 30 min had no significant effect on the membrane potential (-72 ± 4.5 mV) or slope conductance (6.93 ± 1.7 nS) values ($n = 5$; $P > 0.05$; Student's paired *t* test; Fig. 6B). Thus these data indicate that blockade of PP1 and PP2A with okadaic acid neither occludes nor reverses the actions of leptin.

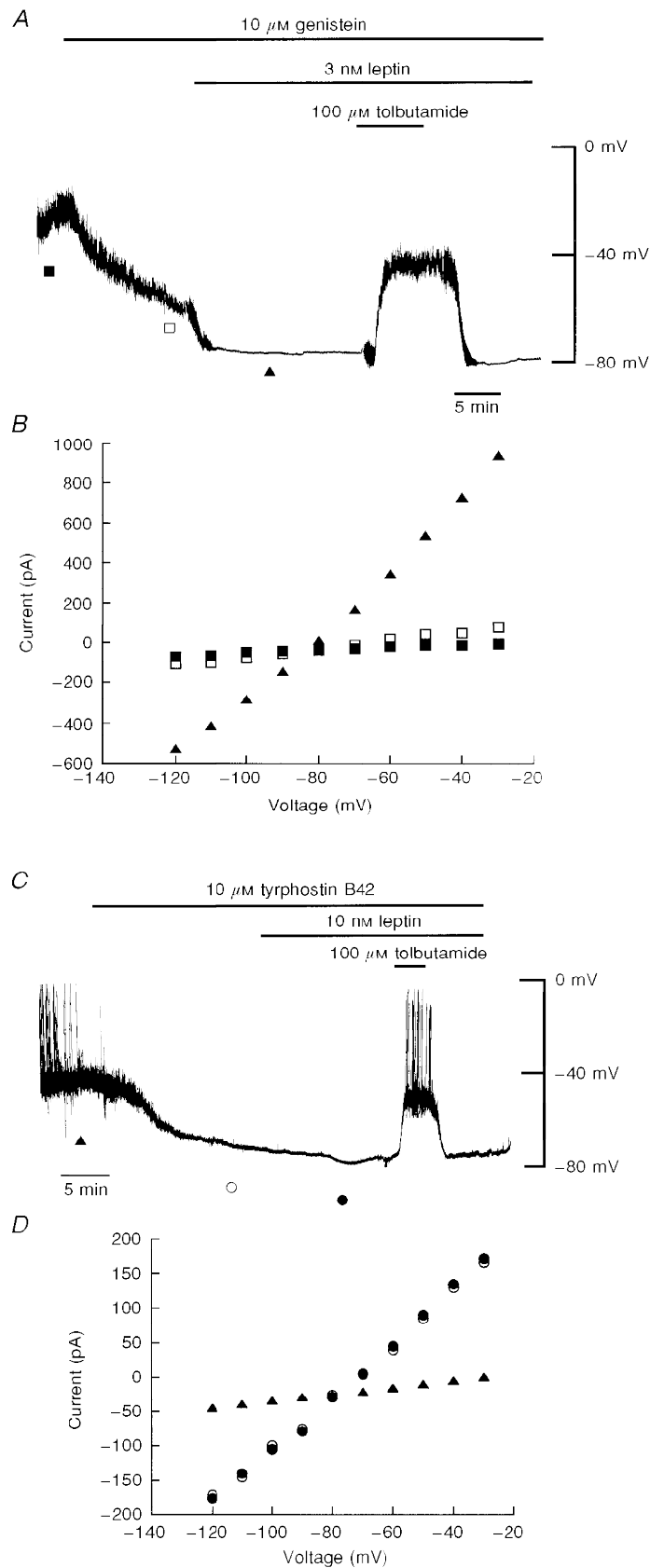
In another series of experiments the effects of the PP2B inhibitor cyclosporin A on leptin activation of K_{ATP} channels were examined. After obtaining the whole-cell configuration and following 15 min dialysis with an electrode solution containing 5 mM ATP and $1 \mu\text{M}$ cyclosporin A, the mean resting membrane potential and slope conductance of CRI-G1 cells were -38 ± 1.6 mV and 0.47 ± 0.1 nS, respectively ($n = 4$). Subsequent application of leptin (10 nM) resulted in hyperpolarization of CRI-G1 cells to -68 ± 4.3 mV with an associated increase in slope conductance to 4.71 ± 0.79 nS ($n = 4$; Fig. 6C). The reversal potential associated with this increase in conductance was -80 ± 1.3 mV, indicating an increase in K^+ conductance ($n = 4$). Together these data indicate that inhibition of dephosphorylation by serine/threonine-specific protein phosphatases does not prevent leptin activation of K_{ATP} channels.

Inhibition of tyrosine phosphatases prevents leptin activation of K_{ATP} channels

Since serine/threonine-specific phosphatases do not prevent the actions of leptin, the effects of the broad-spectrum protein tyrosine phosphatase inhibitor orthovanadate (Swarup, Cohen & Garbers, 1982) were examined. In order to determine whether inhibition of dephosphorylation by tyrosine phosphatases affects leptin activation of K_{ATP} channels, the cells were dialysed with an electrode solution containing 5 mM ATP and $500 \mu\text{M}$ orthovanadate. Under these conditions, the mean resting membrane potential and

Figure 5. The effects of leptin after exposure to tyrosine kinase inhibitors

A, current clamp trace of a cell dialysed with 5 mM ATP. Application of genistein (10 μ M) hyperpolarized the cell from -38 to -60 mV. Subsequent application of leptin (3 nM) and genistein (10 μ M) further hyperpolarized the cell membrane potential to -80 mV. Tolbutamide (100 μ M) completely reversed the hyperpolarizations induced by genistein and leptin. *B*, current-voltage relationships for the voltage clamped currents shown in *A*: ■, control; □, genistein; ▲, leptin and genistein. Genistein slightly increased the membrane conductance relative to control. Application of leptin after exposure to genistein resulted in a substantial further increase in K⁺ conductance. *C*, current clamp trace of a cell dialysed with 5 mM ATP. Application of tyrphostin B42 (10 μ M) hyperpolarized the CRI-G1 cells to -76 mV. Application of leptin (10 nM) after exposure to tyrphostin B42 failed to affect further the cell membrane potential. *D*, current-voltage relationships for the voltage clamped currents obtained at the times indicated in *C*: ▲, control; ○, tyrphostin B42; ●, leptin and tyrphostin B42. Tyrphostin B42 increased the membrane conductance relative to control, with a reversal potential of -79 mV. Subsequent application of leptin in the presence of tyrphostin B42 failed to increase the membrane conductance.



slope conductance of CRI-G1 cells were -42 ± 1.4 mV and 0.60 ± 0.07 nS, respectively ($n = 5$). Application of leptin (10 nM) following dialysis for at least 20 min failed to result in membrane hyperpolarization or an increase in the slope conductance in four of the five cells examined (Fig. 7). Thus the membrane potential and slope conductance values obtained 20 min after leptin application were -46 ± 4.7 mV

and 0.98 ± 0.32 nS, respectively ($n = 5$; $P > 0.05$; Fig. 7). In another three cells, dialysis with the tyrosine phosphatase inhibitor orthovanadate ($500 \mu\text{M}$) prevented the tyrphostin B42-induced hyperpolarization and increased K^+ conductance (data not illustrated), indicating that the effects of the tyrosine kinase inhibitors on K_{ATP} channels are also ultimately due to dephosphorylation. Together these

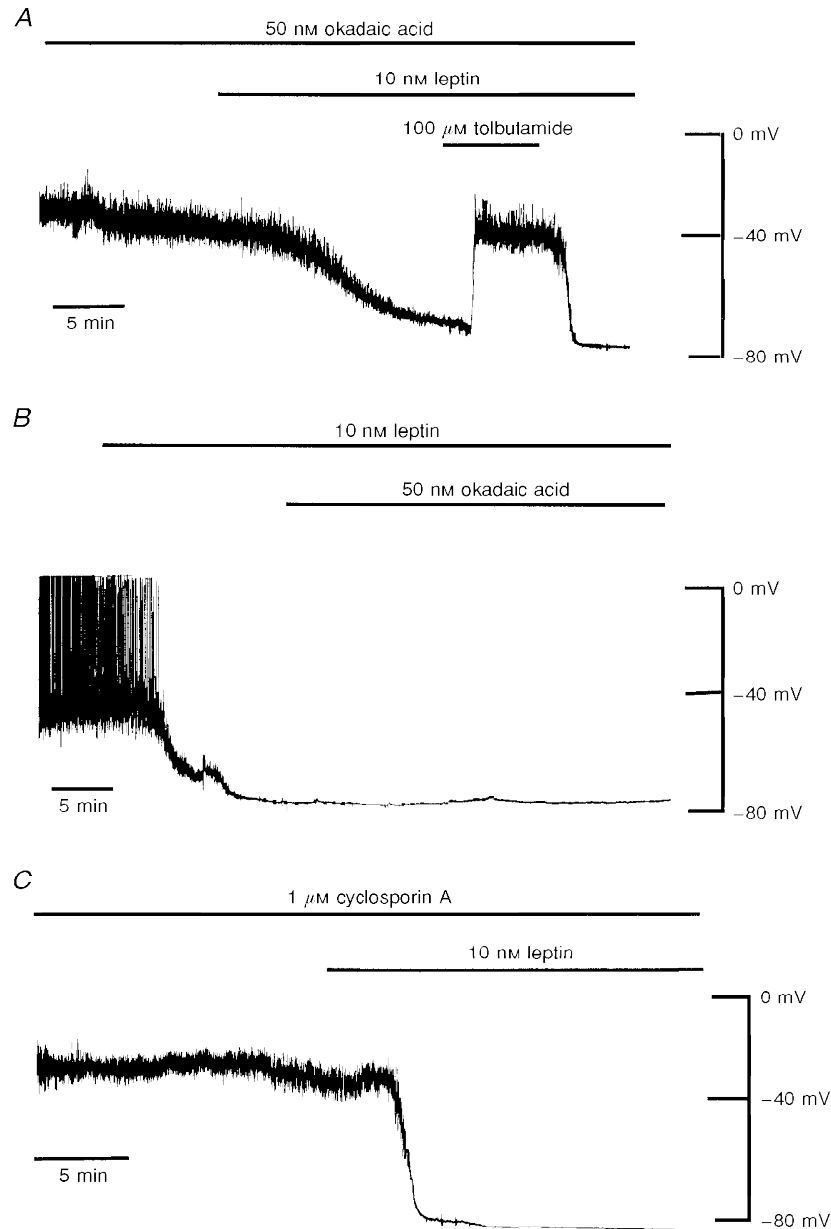


Figure 6. Serine/threonine-specific phosphatase inhibitors do not prevent the actions of leptin

A, current-clamp trace of a cell dialysed with 5 mM ATP. Application of okadaic acid (50 nM) for 15–20 min had no effect on the resting membrane potential of CRI-G1 cells. Addition of leptin (10 nM) after prior exposure to okadaic acid resulted in hyperpolarization of CRI-G1 cells to -78 mV. Tolbutamide ($100 \mu\text{M}$) reversed the leptin-induced hyperpolarization to control levels. *B*, current-clamp record of another cell dialysed with 5 mM ATP. Leptin (10 nM) applied for the time indicated hyperpolarized the cell membrane from -41 to -77 mV. Okadaic acid (50 nM) was unable to reverse the activation of K_{ATP} channels by leptin. *C*, current-clamp record of a cell dialysed with 5 mM ATP and $1 \mu\text{M}$ cyclosporin A. Dialysis of cells with cyclosporin A ($1 \mu\text{M}$) for at least 15 min had no effect on the resting membrane potential and did not prevent the leptin (10 nM)-induced hyperpolarization.

data indicate that inhibition of tyrosine phosphatases by orthovanadate and therefore inhibition of dephosphorylation prevents leptin and tyrphostin B42 activation of K_{ATP} channels.

Wortmannin occludes the actions of tyrphostin B42

We have shown previously that leptin activates K_{ATP} channels in CRI-G1 cells via a PI 3-kinase-dependent process, as wortmannin, a specific inhibitor of PI 3-kinase (Powis *et al.* 1994), inhibits the actions of leptin (J. Harvey & M. L. J. Ashford, unpublished observations). In order to determine whether inhibition of the tyrosine kinases (and therefore tyrosine dephosphorylation) occurs prior to or downstream of PI 3-kinase activation, the effects of wortmannin on tyrphostin B42-induced activation of K_{ATP} channels were examined (Fig. 8). Under current clamp conditions with 5 mM ATP in the electrode solution, application of wortmannin (10 nM) to the bath for 15–20 min failed to affect the membrane potential (-38 ± 2.0 mV; $n = 4$) or slope conductance (0.58 ± 0.08 nS; $n = 4$). After at least 20 min exposure to the PI 3-kinase inhibitor (10 nM),

coapplication of wortmannin (10 nM) and tyrphostin B42 (10 μ M) had no effect on the membrane potential (-39 ± 2.3 mV) or slope conductance (0.61 ± 0.07 nS) values ($n = 4$; $P > 0.05$; Student's paired *t* test; Fig. 8). These data indicate that inhibition of tyrosine phosphorylation by the tyrosine kinase inhibitor tyrphostin B42, leading to activation of K_{ATP} channels, occurs prior to the activation of PI 3-kinase.

DISCUSSION

Recently, we and others have shown that in the CRI-G1 insulin-secreting cell line (Harvey *et al.* 1997) and pancreatic β -cells (Keiffer *et al.* 1997) the *ob* gene product leptin activates K_{ATP} channels, an action consistent with suppression of insulin secretion. Peptide modulation of K_{ATP} channels has previously been shown to be mediated by protein kinase A (Quayle *et al.* 1994; Miyoshi & Nakaya, 1995) and protein kinase C (De Weille *et al.* 1989; Kubo, Quayle & Standen, 1997). Protein kinases are also reported to modulate K_{ATP} channel function in insulin-secreting cell

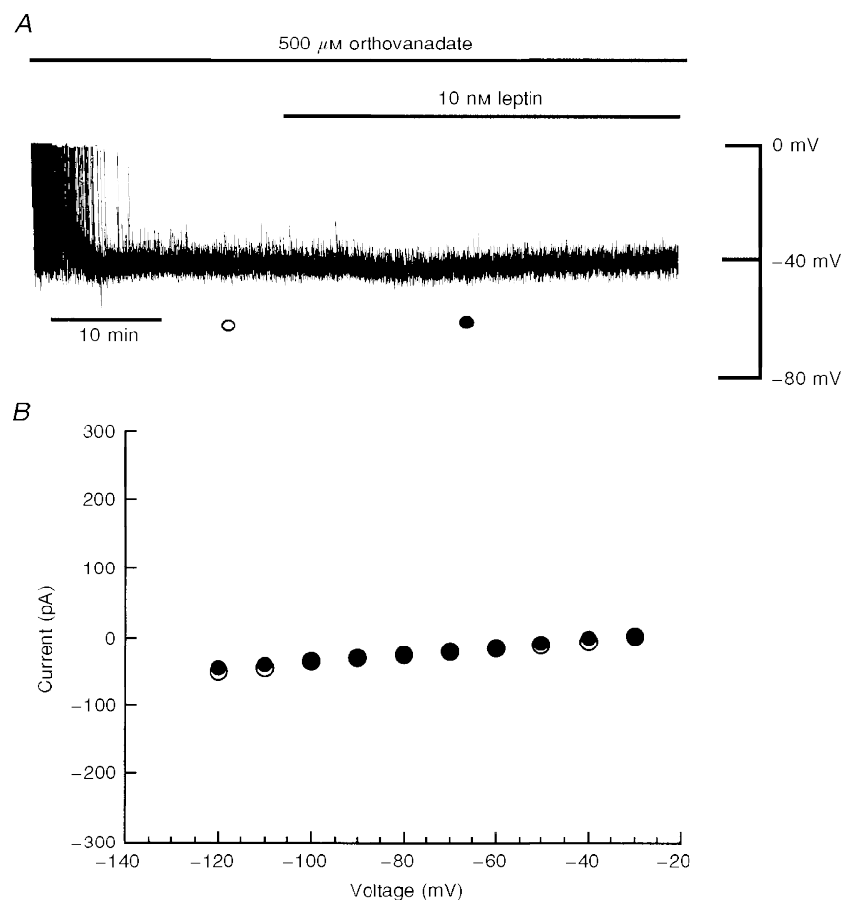


Figure 7. Orthovanadate prevents leptin activation of K_{ATP} channels

A, current clamp record of a cell dialysed with an electrode solution containing 5 mM ATP and 500 μ M orthovanadate. Dialysis with orthovanadate had no effect on the resting membrane potential of CRI-G1 cells; however, it did prevent the leptin-induced hyperpolarization. *B*, plot of current–voltage relationships for voltage clamped macroscopic currents obtained at the times specified in *A*: ○, orthovanadate; ●, orthovanadate and leptin. Orthovanadate blocked the leptin-induced increase in K^+ conductance.

lines (Ribalet *et al.* 1988; Wollheim, Dunne, Peter-Riesch, Bruzzone, Pozzan & Petersen, 1988). In the present study, however, the broad-spectrum protein kinase inhibitor H-7 and the protein phosphatase inhibitors okadaic acid and cyclosporin A had no effect on K_{ATP} channels. Furthermore, these agents failed to block leptin activation of K_{ATP} channels suggesting that protein phosphorylation of serine/threonine residues does not underlie leptin actions in CRI-G1 cells. As the leptin receptor is a member of the class I cytokine receptor family (Tartaglia *et al.* 1995), one likely mechanism by which K_{ATP} channel activity is increased is via activation of tyrosine kinases such as the JAK family (Ihle, 1995). Indeed, the class I cytokine prolactin activates Ca^{2+} -activated K^+ channels via activation of JAK2 in Chinese hamster ovary (CHO) cells (Pervarskaya *et al.* 1995). In contrast, however, our present data are consistent with leptin activation of K_{ATP} channels

involving inhibition of tyrosine kinases and subsequent tyrosine dephosphorylation. Thus, dialysis with AMP-PNP, a non-hydrolysable analogue of ATP, prevented leptin activation of K_{ATP} channels indicating that phosphorylation or dephosphorylation plays a role in this process. In addition, inhibition of tyrosine kinase activity by application of three different tyrosine kinase inhibitors mimicked the leptin-induced hyperpolarization and increase in K^+ conductance. In contrast, the inactive analogues of genistein (daidzein) and tyrphostin B42 (tyrphostin 1) failed to affect the cell membrane potential or slope conductance of CRI-G1 cells. Furthermore, activation of K_{ATP} channels by tyrphostin B42 prevented the actions of leptin suggesting that the mechanism underlying leptin activation of K_{ATP} channels may involve inhibition of tyrosine kinases (and subsequent tyrosine dephosphorylation). Alternatively, in a manner similar to non-

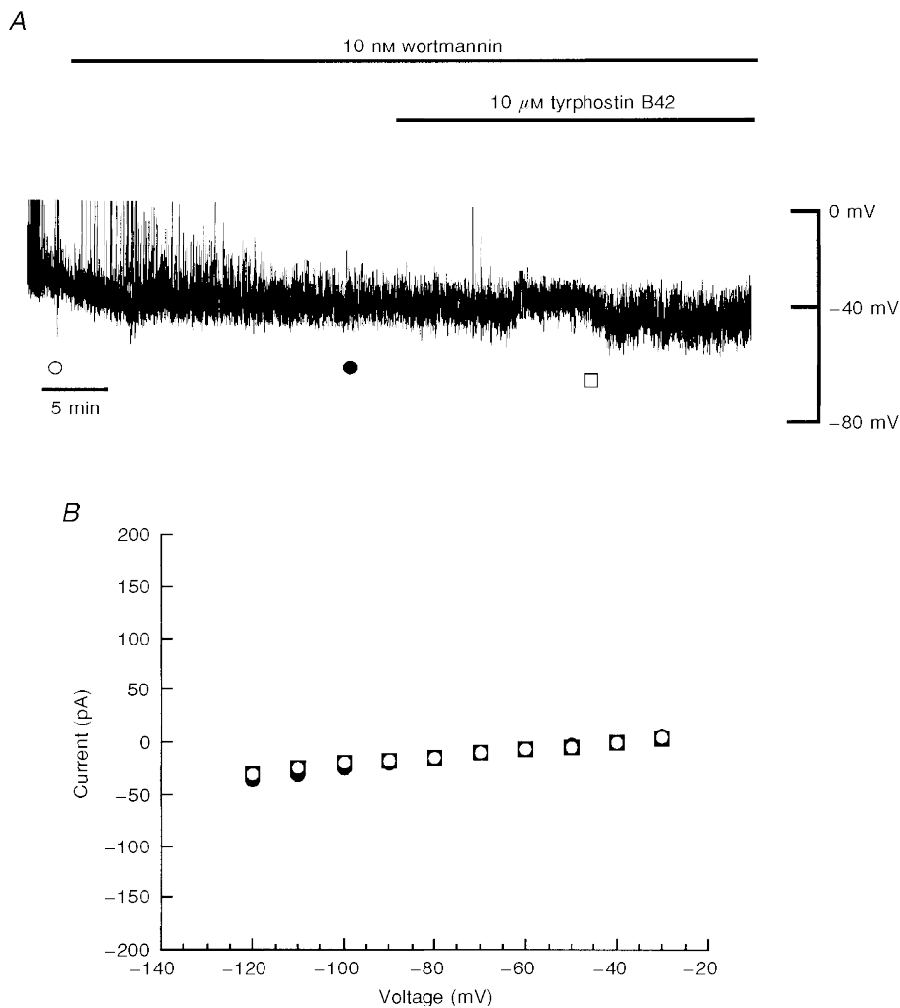


Figure 8. Wortmannin prevents tyrphostin B42 activation of K_{ATP} channels

A, current clamp record of a cell dialysed with 5 mM ATP. Application of wortmannin (10 nM) for the time indicated had no effect on the resting membrane potential. Addition of tyrphostin B42 (10 μ M) after exposure to wortmannin failed to hyperpolarize CRI-G1 cells. *B*, plot of the current–voltage relationships for voltage clamped currents obtained at the points specified in *A*: \circ , control; \bullet , wortmannin; \square , tyrphostin B42 and wortmannin. Wortmannin prevented the tyrphostin-induced increase in K^+ conductance.

selective cation channels in *Aplysia* bag cell neurones (Wilson & Kaczmarek, 1993) and leech mechanosensitive neurones (Catarsi & Drapeau, 1997), the actions of leptin in this study may involve the activation of tyrosine phosphatases. This is further supported by the prevention of leptin activation of K_{ATP} channels and subsequent hyperpolarization following dialysis with the tyrosine phosphatase inhibitor orthovanadate.

In the present study, tyrphostin B42 and herbimycin A were more effective than genistein in activating K_{ATP} channels as both these agents hyperpolarized CRI-G1 cells to more negative potentials and produced a greater increase in K^+ conductance than genistein. Furthermore, genistein, in contrast to tyrphostin B42, did not completely occlude leptin activation of K_{ATP} channels. This is most probably attributable to differences in the specificity or potency of these inhibitors for distinct tyrosine kinases present in these cells. Further studies are required to elucidate the identity of the tyrosine kinase(s) and phosphatase(s) responsible for modulation of this pathway.

In cell-attached recordings, K_{ATP} channels were opened by tyrphostin B42 only when this agent was present in the electrode solution. Similarly, leptin opened K_{ATP} channels in the cell-attached configuration when the protein was in direct contact with the extracellular surface of the membrane patch (Harvey *et al.* 1997). Thus these data suggest that leptin and tyrphostin B42 open K_{ATP} channels in a membrane-delimited manner. In a manner similar to leptin (J. Harvey & M. L. J. Ashford, unpublished observations), the effects of tyrphostin B42 were blocked by the PI 3-kinase inhibitor wortmannin, suggesting that the mechanism underlying leptin activation of K_{ATP} channels in the CRI-G1 insulin-secreting cell line involves tyrosine dephosphorylation of unidentified cytosolic proteins prior to activation of PI 3-kinase. Recent studies indicate that the insulin receptor substrate proteins, in particular IRS-1, are capable of activating PI 3-kinase following activation of cytokine receptors including insulin (Myers & White, 1996) and prolactin (Berlanga *et al.* 1997). Furthermore, leptin has been shown to downregulate tyrosine phosphorylation of IRS-1 by insulin in hepatocytes (Cohen, Novick & Rubinstein, 1996). Thus, IRS-1 is one possible target site for dephosphorylation by leptin in this cell line. However, further biochemical experiments are required to determine the protein(s) that is dephosphorylated following leptin receptor activation.

The modulatory action of inhibitors of tyrosine kinases and phosphatases on K_{ATP} channel function observed in the present study parallels the actions of these agents on the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel. Thus genistein activates the CFTR chloride channel in a number of expression systems (Illek, Fischer, Santos, Widdicombe, Machen & Reenstra, 1995; Yang, Cheng, Wang, Price & Hwang, 1997), whereas orthovanadate inhibits genistein-induced channel activation (Illek *et al.* 1995). Tyrosine kinase inhibitors are also

reported to increase the activity of other channel types including Ca^{2+} -activated K^+ channels in smooth muscle cells (Xiong, Burnette & Cheung, 1995) and hippocampal neurones (Abdul-Ghani, Valiante, Carlen & Pennefather, 1996) and cation channels in leech pressure-sensitive neurones (Aniksztejn, Catarsi & Drapeau, 1997). In contrast, the activity of voltage-dependent Ca^{2+} channels is reduced by tyrosine kinase inhibitors in GH_3 pituitary cells (Cataldi *et al.* 1996) and dorsal root ganglion neurones (Fitzgerald & Dolphin, 1997), whereas orthovanadate increases the activity of these channels (Cataldi *et al.* 1996). Therefore, like numerous other channel types, the activity of K_{ATP} channels is influenced by the interplay between protein tyrosine kinase and tyrosine phosphatase activity.

The involvement of tyrosine dephosphorylation in leptin activation of K_{ATP} channels in CRI-G1 cells contrasts with the role of tyrosine phosphorylation proposed for prolactin activation of Ca^{2+} -activated K^+ channels in CHO cells (Prevarskaya *et al.* 1995), as activation of JAK2 is implicated in the stimulatory action of prolactin. Since prolactin, like leptin, can activate PI 3-kinase (Berlanga *et al.* 1997), differences in the cellular signalling pathways downstream of PI 3-kinase may explain the opposing roles of tyrosine phosphorylation underlying the actions of these cytokine receptors. In support of this, different isoforms of PI 3-kinase have been identified (Zvelebil *et al.* 1996), which may activate separate but interesting pathways. Furthermore, this may explain why insulin prevents leptin activation of K_{ATP} channels in CRI-G1 insulinoma cells (J. Harvey & M. L. J. Ashford, unpublished observations), even though both these agents activate PI 3-kinase. Alternatively, the basal phosphorylation state of target proteins (or channels) and the number or type (tyrosine) of possible phosphorylation sites may be important.

Leptin has recently been shown to activate K_{ATP} channels in hypothalamic glucose-receptive neurones (Spanswick, Smith, Groppi, Logan & Ashford, 1997), an action that may reflect its antiobesity actions in the whole animal. It will obviously be interesting to determine whether the leptin receptor transduction pathway(s) in the brain is identical to that described here or whether significant alternative mechanisms are present in other cell types. There is increasing evidence that a feedback loop exists between adipose tissue and pancreatic β -cells such that administration of insulin increases leptin expression (Mizuno *et al.* 1996) and application of leptin reduces plasma levels of insulin (Pellemounter *et al.* 1995; Halaas *et al.* 1995). However, in the obese state this feedback loop is dysfunctional, such that obese individuals show resistance to leptin even under conditions of hyperleptinaemia. In the absence of any defect in leptin production or the leptin receptor, leptin resistance is likely to occur downstream of the receptor. In this respect, the involvement of tyrosine dephosphorylation prior to PI 3-kinase stimulation in leptin activation of K_{ATP} channels may be important in understanding the signalling processes underlying these defects.

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