The essential role of phosphoinositide 3-kinase in leptin-induced \( K_{ATP} \) channel activation in the rat CRI-G1 insulinoma cell line

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The mechanism by which leptin increases ATP-sensitive \( K^+ \) (\( K_{ATP} \)) channel activity was investigated using the insulin-secreting cell line, CRI-G1. Wortmannin and LY 294002, inhibitors of phosphoinositide 3-kinase (PI3-kinase), prevented activation of \( K_{ATP} \) channels by leptin. The inositol phospholipids phosphatidylinositol bisphosphate and phosphatidylinositol trisphosphate (PtdIns(3,4,5)\( P_3 \)) mimicked the effect of leptin by increasing \( K_{ATP} \) channel activity in whole-cell and inside-out current recordings. LY 294002 prevented phosphatidylinositol bisphosphate, but not PtdIns(3,4,5)\( P_3 \), from increasing \( K_{ATP} \) channel activity, consistent with the latter lipid acting as a membrane-associated messenger linking leptin receptor activation and \( K_{ATP} \) channels. Signaling cascades, activated downstream from PI 3-kinase, utilizing PtdIns(3,4,5)\( P_3 \) as a second messenger and commonly associated with insulin and cytokine action (MAPK, p70 ribosomal protein-S6 kinase, stress-activated protein kinase 2, MAPK, and protein kinase B), do not appear to be involved in leptin-mediated activation of \( K_{ATP} \) channels in this cell line. Although PtdIns(3,4,5)\( P_3 \) appears a plausible and attractive candidate for the messenger that couples \( K_{ATP} \) channels to leptin receptor activation, direct measurement of PtdIns(3,4,5)\( P_3 \) demonstrated that insulin, but not leptin, increased global cellular levels of PtdIns(3,4,5)\( P_3 \). Possible mechanisms to explain the involvement of PI 3-kinases in \( K_{ATP} \) channel regulation are discussed.

The hormone leptin, secreted by adipocytes, has a major influence on body weight homeostasis (1, 2). Although the hypothalamus is considered the main target for leptin, particularly with respect to body weight regulation, it is clear that this hormone has distinct actions on other peripheral, target organs. There have been several reports that leptin reduces secretion from pancreatic beta cells (3–6), although this view is not shared by all investigators (7). One mechanism proposed to explain the leptin-induced reduction in insulin secretion is via activation of ATP-sensitive \( K^+ \) (\( K_{ATP} \)) channels (8, 9). This increase in potassium current results in beta cell hyperpolarization, reduced calcium entry, and hence decreased insulin secretion. In addition, there are features common to both insulin-secreting cells and leptin-sensitive hypothalamic neurones (10, 11), most notably glucose responsiveness and the presence of \( K_{ATP} \) channels, which are activated by exposure of the cells to leptin. The apparent involvement of both leptin receptors and \( K_{ATP} \) channel activation in key systems involved in metabolic homeostasis has led us to examine the likely signal transduction pathways underlying this effect.

The leptin receptor belongs to the class I cytokine receptor superfamily (1, 2), members of which are thought to signal via janus-tyrosine kinases. Activated janus-tyrosine kinases can mediate signaling via insulin receptor substrate proteins (12–14), following tyrosine phosphorylation become docking sites for Src homology 2-containing enzymes like phosphoinositide 3-kinase (PI 3-kinase). Indeed, leptin is reported to stimulate glucose transport in C2C12 myotubules via a PI 3-kinase-dependent process (15), and leptin signaling in these cells employs janus-tyrosine kinase 2 and insulin receptor substrate 2 in this process (16). There is also growing evidence that PI 3-kinase plays a pivotal role in the signal transduction pathways linking insulin receptor activation and various cellular responses (14). Wortmannin and LY 294002 are inhibitors of PI 3-kinase (17, 18) that are reported to inhibit insulin-induced activation of protein kinase B (PKB) (19), mitogen-activated protein kinase (MAPK) (20, 21), and p70\(^{S6K} \) (22). We have shown recently that leptin activation of \( K_{ATP} \) channels in CRI-G1 cells is sensitive to both wortmannin and LY 294002, suggesting the possible involvement of a PI 3-kinase and that insulin occludes leptin activation of \( K_{ATP} \) channels (23), indicating cross-talk between insulin and leptin-stimulated signaling pathways. Thus, the cell-signaling pathways initiated by leptin action in CRI-G1 cells may overlap with those of insulin. In support of this possibility, recent reports indicate that leptin-induced proliferation involves activation of MAPK in C3H10T1/2 cells (24) and the insulin-secreting MIN6 cell line (25). It has also been demonstrated (26) that in Chinese hamster ovary cells stably expressing leptin receptor isoforms, tyrosine phosphorylation of MAPK is enhanced. Most of the cellular roles of PI 3-kinase have been attributed to the lipid products of these enzymes, which bind to target molecules via specific lipid binding domains. There is mounting evidence that phosphatidylinositol trisphosphate (PtdIns(3,4,5)\( P_3 \)) levels increase in cells following stimulation by a variety of agonists and that the increased PtdIns(3,4,5)\( P_3 \) levels precede activation of downstream signaling molecules (27).

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1 The abbreviations used are: PI 3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B; MAPK, mitogen-activated protein kinase; PtdIns(3,4,5)\( P_3 \), phosphatidylinositol trisphosphate; PtdIns(4,5)\( P_2 \), phosphatidylinositol bisphosphate; PLC, phospholipase C; p70\(^{S6K} \), p70 ribosomal protein-S6 kinase.
The possibility that phosphoinositides are involved in signaling to potassium channels is indicated by numerous reports suggesting that phosphatidylinositol bisphosphate (PtdIns(4,5)P2) can directly activate cloned inward rectifier K⁺ channels (28) and native (29, 30) and cloned (consisting of the inward rectifier channel, Kir6.2, and the sulfonylurea receptor, SUR1 subunits) K⁺ATP channels (31, 32). In this report we compare the effect of leptin action on K⁺ATP channel activity in CRI-G1 cells with that induced by PtdIns(4,5)P2 or PtdIns(3,4,5)P3 using whole-cell and inside-out current recordings.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Cells from the rat insulin-secreting cell line, CRI-G1, were grown in Dulbecco’s modified Eagle’s medium with sodium pyruvate (0.01%) and glucose (0.1%) (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 2–3 days as described previously (33), 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 recordings to monitor membrane potential with excursions to voltage clamp mode to examine macroscopic currents and excised inside-out recordings to examine single channel responses, as described previously (9). During voltage clamp recordings, the membrane potential was held at −50 mV, and 10 mV steps of 100 ms duration were applied every 200 ms (range clamp recordings, the membrane potential was held at −50 mV, and 10 mV steps of 100 ms duration were applied every 200 ms (range 60–120 mV). Current and voltage were measured using an Axopatch 200 B amplifier (Axon Instruments), and currents evoked in response to the voltage step protocol were analyzed 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 analyzed for current amplitude and channel activity (NfPo, where N is the number of functional channels and Po is the open probability) as described previously (34). All data are expressed as mean ± S.E., 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 MΩ for whole cell recordings and 8–12 MΩ for inside-out experiments when filled with electrolyte solution. The pipette solution for whole-cell recordings comprised 140 mM KCl, 0.6 mM MgCl₂, 2.73 mM CaCl₂, 5.0 mM ATP, 10 mM EGTA, 10 mM HEPES, pH 7.2 (free [Ca²⁺] of 100 nM), whereas for single channel recordings the pipette solution contained: 140 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, pH 7.2. The bath solution for whole cell recordings comprised of normal saline: 135 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 25 mM HEPES, pH 7.4, whereas for inside-out experiments the bath solution contained: 140 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM EGTA, 10 mM HEPES, pH 7.2 (free Ca²⁺ of 30 nM). The free Ca²⁺ concentrations were calculated using the “METLIG” program (P. England and R. Denton, University of Bristol, UK). 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).

**Determination of PKB Activity**—CRI-G1 cells were deprived of serum overnight in Dulbecco’s modified Eagle’s medium and then stimulated for various times at 37°C with either insulin (100 nM) or leptin (10 nM) in the presence or absence of 100 μM wortmannin. Each 10-cm dish of cells was lysed in 1 ml of ice-cold lysis buffer (50 mM Tris·HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% v/v Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 1 μM micromycin-LR, 0.27 mM sucrose, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 0.1% v/v 2-mercaptoethanol). The lysates were centrifuged at 4°C for 5 min at 13,000 × g, and 100 μg of each lysate was incubated for 60 min with 5 μl of protein G-Sepharose coupled to 5 μl of PKB isoform antibodies (35). The protein G-Sepharose-antibody-PKB complex was washed twice with 1 ml of lysis buffer containing 0.5% NaCl and twice with 50 mM Tris·HCl, pH 7.5, 0.1 mM EGTA, and 0.1% (v/v) 2-mercaptoethanol. The immunoprecipitates were assayed for PKB activity using Cross-tide as a substrate (36). One unit of activity is defined as the amount, which catalyzes the incorporation of 1 nmol of phosphate into 1 min. Each experimental assay was performed 3 times, in triplicate.

**Phosphatidylinositol(3,4,5)P3 Triphosphate Measurement**—PtdIns(3,4,5)P3 levels were determined by radioligand displacement assay (37, 38). Briefly, CRI-G1 cells were cultured in 80-cm² flasks as described previously. The cell monolayer was rinsed with normal saline prior to incubation with either insulin (100 nM) or leptin (10 nM) all prepared in normal saline containing 0.2% bovine serum albumin. After 30 min the cells were aspirated, and the cells were washed with 10% trichloroacetic acid. The trichloroacetic acid pellet was harvested by scraping and collected by centrifugation for 2–5 min at 13,000 rpm in a microfuge. The pellet was pre-extracted with neutral chloroform/methanol (1:2 v/v) to remove polar molecules and inositol phosphates, and then lipids were isolated by Bligh & Dyer extraction with chloroform/methanol (4:90:1 v/v). The isolated lipids were vacuum dried and stored at −80°C until assayed, after alkaline hydrolysis to release lipid headgroups, by radioligand displacement of inositol 1,3,4,5-tetrakisphosphate as described previously (37, 38). The remaining protein was vacuum-dried and resuspended in 1 mM NaOH, and the protein content was assayed using the DC protein assay kit (Bio-Rad). Results were expressed as pmol PtdIns(3,4,5)P3 mg⁻¹ protein, and each assay was carried out a minimum of nine times.

**Drugs**—Tolbutamide, rapamycin, Mg-ATP, U71322, insulin, and wortmannin were obtained from Sigma. Recombinant human leptin, LY 294002, and PtdIns(4,5)P2 were obtained from Calbiochem. PD 98059 was a gift from Professor John Hughes, Parke Davis Neuroscience Research Center (Cambridge, UK). SB 203580 was a gift from Professor P. Cohen, University of Dundee, Scotland. Tolbutamide was made up as a stock solution in 100 mM stock solution in Me₂SO. Mg-ATP was made up as a 100 mM stock solution in 10 mM stock solution of MgSO₄, whereas LY 294002 was stored as 10 mM stock solution in 1% methanol. PtdIns(4,5)P2 and PtdIns(3,4,5)P3 were stored as 1 mM stock solutions in distilled water and sonicated before use. PD 98059 and SB 203580 were stored as a 10 mM stock solutions in Me₂SO₄, whereas rapamycin was prepared as a 100 μM stock solution in Me₂SO₄. U73122 was stored as a 2 mM stock solution in 1% ethanol.

**RESULTS**

**Leptin Activates K⁺ATP Channels in CRI-G1 Cells**—Under current clamp conditions with 5 mM ATP in the pipette solution to maintain K⁺ATP channels in the closed state, the mean resting membrane potential of CRI-G1 cells was −37.0 ± 1.3 mV (n = 4). Application of leptin (10 nM) hyperpolarized the CRI-G1 cells to −64.8 ± 3.2 mV (n = 4; Fig. 1A). Examination of the voltage-clamped macroscopic currents indicates that prior to the addition of leptin the slope conductance of the cells was 0.65 ± 0.09 nS, and following exposure to leptin (10 nM), this increased to 2.23 ± 0.64 nS (n = 4; Fig. 1B). The reversal potential (obtained from the point of intersection of the current-voltage relationships) associated with the leptin-induced conductance increase was −77.8 ± 2.1 mV (n = 4), which is close to the calculated value for E_K of −84 mV in this system, indicating an increase in K⁺ conductance. Application of tolbutamide (100 μM) completely reversed the leptin-induced hyperpolarization and decreased conductance to pre-leptin levels (n = 3), indicating that leptin activates K⁺ATP channels in this cell line. Application of inhibitors of PI 3-kinase, wortmannin (10 nM; n = 5; data not shown) and LY294002 (10 μM; n = 5; Fig. 1, C and D) had no significant affect on the resting membrane potential or slope conductance of CRI-G1 cells but prevented the leptin-induced cell hyperpolarization and activation of K⁺ATP currents. These data are in agreement with our previous studies demonstrating that leptin activates K⁺ATP channels in the rat CRI-G1 insulinoma cell line (9) presumably via activation of PI 3-kinase (23).

**PtdIns(4,5)P2 Mimics Leptin Activation of K⁺ATP Channel Currents**—There have been several studies demonstrating that phosphoinositides, most particularly, PtdIns(4,5)P2 can directly regulate the activity of various ion transporters and channels, including members of the inward rectifying family of K⁺ channels, to which the K⁺ATP channel belongs (29–32). Consequently, CRI-G1 cells were dialyzed with an electrode solu-
Leptin activates \( K_{\text{ATP}} \) channel currents via a PI 3-kinase-dependent process. A, representative whole cell current clamp recording of a CRI-G1 cell dialyzed with an electrode solution containing 5 mM ATP. In this and subsequent figures the trace begins approximately 5 min after obtaining the whole cell configuration. Application of leptin (10 nM) for the time indicated resulted in hyperpolarization of the membrane from -41 mV to -76 mV, an action readily reversed by the sulfonylurea tolbutamide (100 \( \mu M \)). Note that there is a delay of around 2 min from the time of drug addition until complete bath exchange.

Application of leptin (10 nM) after exposure to LY 294002 failed to hyperpolarize CRI-G1 cells. D, plot of the current-voltage relationships for voltage-clamped currents obtained at the points specified in C: LY 294002 (○) and LY 294002 and leptin (●). LY 294002 prevented the leptin-induced increase in \( K^+ \) conductance.

Leptin increases the membrane conductance relative to control and tolbutamide reversed the leptin-induced increase in conductance with a reversal potential of -76 mV. C, whole cell current clamp record of a CRI-G1 cell dialyzed with 5 mM ATP. Note that under these recording conditions CRI-G1 cells do not always exhibit spontaneous action potentials.

Application of leptin (10 nM) resulted in hyperpolarization and an increased slope conductance. Tolbutamide (100 \( \mu M \)) readily reversed the leptin-induced hyperpolarization and increase in \( K^+ \) conductance.

These data indicate that, in a manner similar to leptin, PtdIns(4,5)P\(_2\) activates \( K_{\text{ATP}} \) channels in CRI-G1 insulin-secreting cells. This was confirmed by examination of the actions of PtdIns(4,5)P\(_2\) (1 \( \mu M \)) on \( K_{\text{ATP}} \) channel activity when applied directly to inside-out membrane patches (Fig. 2E). PtdIns(4,5)P\(_2\) induced activation of channel activity in the presence of ATP with values for \( N_P \) of 1.09 ± 0.28, 0.03 ± 0.01, and 0.65 ± 0.21 (\( n = 3 \)) in control, 0.1 mM MgATP, and MgATP + 1 \( \mu M \) PtdIns(4,5)P\(_2\), respectively.

PtdIns(4,5)P\(_2\) is the precursor for the well studied phospholipase C (PLC)-linked second messengers, inositol 1,4,5-triphosphate and diacylglycerol, and as such it is possible that leptin-induced \( K_{\text{ATP}} \) channel activation may be attributed to a PLC-dependent process. Therefore, the effects of the PLC inhibitor, U73122, were investigated on leptin-induced \( K_{\text{ATP}} \) current activation. Under current clamp conditions with 5 mM ATP and 2 \( \mu M \) U73122 present in the electrode solution, the mean resting membrane potential and slope conductance of CRI-G1 cells were -35.4 ± 0.85 mV and 0.61 ± 0.09 nS, respectively (\( n = 4 \)). Subsequent application of leptin (10 nM) resulted in hyperpolarization and an increased slope conductance to -68.2 ± 4.0 mV and 4.07 ± 0.96 nS, respectively (\( n = 4 \); data not shown) with an associated reversal potential of -79.0 ± 1.1 mV (\( n = 4 \)) indicating the involvement of a \( K^+ \) conductance. Tolbutamide (100 \( \mu M \)) readily reversed the leptin-induced hyperpolarization and increase in \( K^+ \) conductance to pre-leptin levels (-38.2 ± 4.2 mV and 0.52 ± 0.02 nS (\( n = 4 \))). Therefore it is unlikely that a phosphoinositide-specific PLC is involved in leptin activation of \( K_{\text{ATP}} \) channels.

LY 294002 Prevents PtdIns(4,5)P\(_2\) Activation of \( K_{\text{ATP}} \) Channel Currents—Stimulation of cells with insulin or certain growth factors results in the activation of a Type I PI 3-kinase, which primarily phosphorylates PtdIns(4,5)P\(_2\) to PtdIns(3,4,5)P\(_3\) (14). Because PtdIns(4,5)P\(_2\) is a key substrate for PI 3-kinase and inhibitors of this enzyme system prevent leptin activation of \( K_{\text{ATP}} \) channels (23), we have examined the sensitivity of the PtdIns(4,5)P\(_2\)-mediated increase in \( K_{\text{ATP}} \) current to PI 3-kinase inhibitors. Cells were incu-
bated with LY 294002 (10 μM) for 25–30 min prior to obtaining the whole-cell configuration and subsequent dialysis with 5 mM ATP and 50 μM PtdIns(4,5)P₂. Dialysis with PtdIns(4,5)P₂ resulted in a slowly developing hyperpolarization of the cell membrane to −72 mV, an action readily reversed by the sulfonylurea, tolbutamide (100 μM). B, plot of the current voltage relations for the currents obtained in A: ○, control (before any change in resting membrane potential); □, PtdIns(4,5)P₂; and ○, tolbutamide. PtdIns(4,5)P₂ increased the membrane conductance relative to control and tolbutamide reversed the PtdIns(4,5)P₂-induced increase in conductance with a reversal potential of −83 mV. C, current clamp trace of a cell dialyzed with 5 mM ATP and 50 μM PtdIns(4,5)P₂. Incubation of cells with LY 294002 (10 μM) for at least 20 min prior to obtaining the whole cell configuration prevented the PtdIns(4,5)P₂-induced hyperpolarization. D, plot of the current voltage relations for the currents obtained in C: LY 294002 (●) and LY 294002 and PtdIns(4,5)P₂ (○). E, inside-out recordings of single K<sub>ATP</sub> channel activity at a membrane potential of −40 mV. Application of 0.1 mM Mg-ATP markedly reduced channel activity. Addition of 1 μM PtdIns(4,5)P₂ in the presence of Mg-ATP elicited a substantial activation of K<sub>ATP</sub> channel activity. F, identical experiment to E using a separate patch that was exposed to 10 μM LY 294002. Note that the addition of 1 μM PtdIns(4,5)P₂ in the presence of LY 294002 does not induce an increase in K<sub>ATP</sub> channel activity.

Fig. 2. PtdIns(4,5)P₂ activates K<sub>ATP</sub> channel currents via a PI 3-kinase-dependent process. A, representative current clamp record of a CRI-G1 cell dialyzed with 5 mM ATP and 50 μM PtdIns(4,5)P₂. Dialysis with PtdIns(4,5)P₂ resulted in a slowly developing hyperpolarization of the cell membrane to −72 mV, an action readily reversed by the sulfonylurea, tolbutamide (100 μM). B, plot of the current voltage relations for the currents obtained in A: ○, control (before any change in resting membrane potential); □, PtdIns(4,5)P₂; and ○, tolbutamide. PtdIns(4,5)P₂ increased the membrane conductance relative to control and tolbutamide reversed the PtdIns(4,5)P₂-induced increase in conductance with a reversal potential of −83 mV. C, current clamp trace of a cell dialyzed with 5 mM ATP and 50 μM PtdIns(4,5)P₂. Incubation of cells with LY 294002 (10 μM) for at least 20 min prior to obtaining the whole cell configuration prevented the PtdIns(4,5)P₂-induced hyperpolarization. D, plot of the current voltage relations for the currents obtained in C: LY 294002 (●) and LY 294002 and PtdIns(4,5)P₂ (○). E, inside-out recordings of single K<sub>ATP</sub> channel activity at a membrane potential of −40 mV. Application of 0.1 mM Mg-ATP markedly reduced channel activity. Addition of 1 μM PtdIns(4,5)P₂ in the presence of Mg-ATP elicited a substantial activation of K<sub>ATP</sub> channel activity. F, identical experiment to E using a separate patch that was exposed to 10 μM LY 294002. Note that the addition of 1 μM PtdIns(4,5)P₂ in the presence of LY 294002 does not induce an increase in K<sub>ATP</sub> channel activity.

Leptin Signaling to K<sub>ATP</sub> Channels in Insulinoma Cells
(N\textsubscript{P\textsubscript{o}}) was 1.00 ± 0.39 in control, 0.05 ± 0.01 in the presence of 10 μM LY 294002 + 0.1 mM Mg-ATP, and 0.03 ± 0.01 (n = 3) upon further addition of 1 μM PtdIns(4,5)P\textsubscript{2}. These data demonstrate that blockade of PI 3-kinase prevents PtdIns(4,5)P\textsubscript{2} activation of K\textsubscript{ATP} channels in these cells, indicating that this molecule does not directly mediate leptin activation of K\textsubscript{ATP} channels.

**PtdIns(3,4,5)P\textsubscript{3} Activates K\textsubscript{ATP} Channel Currents**—As both PtdIns(3,4,5)P\textsubscript{3} and leptin-induced activation of K\textsubscript{ATP} channels is prevented by PI 3-kinase inhibitors, we examined whether PtdIns(3,4,5)P\textsubscript{3} was the signaling molecule. Consequently, cells were dialyzed with an electrode solution containing 5 mM ATP and 1–20 μM PtdIns(3,4,5)P\textsubscript{3}. Immediately after obtaining the whole-cell configuration the mean resting membrane potential and slope conductance of the cells was −36.5 ± 3.2 mV and 0.72 ± 0.05 nS, respectively (n = 4), and 6–11 min after dialysis (Fig. 3, A and B) with 20 μM PtdIns(3,4,5)P\textsubscript{3}, the cells hyperpolarized to −64.7 ± 1.7 mV, with an increased slope conductance to 1.95 ± 0.29 nS (n = 4). Tolbutamide (100 μM) completely reversed the PtdIns(3,4,5)P\textsubscript{3}-induced hyperpolarization and increase in K\textsuperscript{+} conductance to −37.2 ± 2.8 mV and 0.61 ± 0.09 nS, respectively (n = 4), indicating the involvement of K\textsubscript{ATP} channels. In addition, the effects of direct application of PtdIns(3,4,5)P\textsubscript{3} on K\textsubscript{ATP} channel activity in excised inside-out patches were examined (Fig. 3E). Following excision into the inside-out configuration, addition of 0.1 mM Mg-ATP to the intracellular surface of the patch produced a marked inhibition of mean channel activity (N\textsubscript{P\textsubscript{o}}) from 2.08 ± 0.85 to 0.30 ± 0.12, respectively (n = 5). Subsequent application of PtdIns(3,4,5)P\textsubscript{3} (1 μM) in the continued presence of 0.1 mM Mg-ATP produced a significant (p < 0.05) and rapid increase in N\textsubscript{P\textsubscript{o}} to 1.55 ± 0.37 (n = 5).

In a separate series of experiments, cells were exposed to LY 294002 (10 μM) for at least 25 min prior to obtaining the whole cell configuration and dialysis with 5 mM ATP and 20 μM PtdIns(3,4,5)P\textsubscript{3}. Within 1–2 min of obtaining the whole cell configuration (Fig. 3, C and D), the resting membrane potential and slope conductance of CRI-G1 cells were −40.3 ± 0.88 mV and 0.59 ± 0.08 nS, respectively (n = 3). However, 5–11 min of dialysis with PtdIns(3,4,5)P\textsubscript{3}, in the presence of LY 294002, resulted in hyperpolarization of the membrane to −61.7 ± 1.2 mV (n = 3; p < 0.05) associated with an increase in slope conductance to 1.62 ± 0.14 nS (n = 3; p < 0.05). Furthermore, the PtdIns(3,4,5)P\textsubscript{3}-induced increase in channel activity was also observed directly in patches exposed to 10 μM LY 294002, where the mean N\textsubscript{P\textsubscript{o}} values were: 0.56 ± 0.24, 0.02 ± 0.01, and 1.30 ± 0.44 for control (n = 3; p < 0.05), 0.1 mM MgATP, and MgATP + 1 μM PtdIns(3,4,5)P\textsubscript{3}, respectively (Fig. 3F). Therefore, in contrast to the findings with PtdIns(4,5)P\textsubscript{2}, the PI 3-kinase product PtdIns(3,4,5)P\textsubscript{3} is a candidate messenger for the direct activation of K\textsubscript{ATP} channels in these cells.

**Effects of Insulin and Leptin on PtdIns(3,4,5)P\textsubscript{3} Levels in CRI-G1 Cells**—To determine whether leptin mediates its effects on K\textsubscript{ATP} channel activity in CRI-G1 cells via enhanced synthesis of PtdIns(3,4,5)P\textsubscript{3}, the levels of this lipid were analyzed. As a positive control, we also examined the effects of insulin on PtdIns(3,4,5)P\textsubscript{3} levels in CRI-G1 cells as we have demonstrated that these cells respond to the presence of 100 nM insulin with a significant increase in PKB activity. Application of 100 nM insulin (30-min exposure) induced a significant increase (Fig. 4) in PtdIns(3,4,5)P\textsubscript{3} levels (p < 0.001). However, 10 nM leptin (cells stimulated for 30 min) had no significant effect on PtdIns(3,4,5)P\textsubscript{3} levels (Fig. 4). Consequently, although leptin-induced activation of K\textsubscript{ATP} channels is via a PI 3-kinase-dependent process and PtdIns(3,4,5)P\textsubscript{3} can clearly activate K\textsubscript{ATP} channels directly, there is no direct correlation between this leptin response and global PtdIns(3,4,5)P\textsubscript{3} levels in this cell line. However, this data does not preclude the possibility that a small and/or localized, increase in PtdIns(3,4,5)P\textsubscript{3} may be elicited by leptin receptor stimulation. Such an increase may not be detected by the global assay but could activate K\textsubscript{ATP} channels directly or be sufficient to activate downstream effectors which in turn could activate K\textsubscript{ATP} channels.

**Effects of Leptin on PKB and p70\textsuperscript{S6K} Activities**—Activation of PI 3-kinase and the subsequent production of 3-phosphorylated inositol lipids is generally sufficient to trigger many insulin-stimulated pathways (14), indicating that this enzyme is situated at the apex of divergent signal transduction cascades. Consequently, we have examined the likelihood that the downstream signaling molecules identified with insulin action are also associated with leptin activation of K\textsubscript{ATP} channels. Recently, evidence has accumulated linking the effects of insulin and other growth factors on cell function with protein kinase B (also known as Akt), which lies downstream of PI 3-kinase (19, 27). It is generally considered that PKB is regulated through activation of PI 3-kinase by a dual mechanism. Direct binding of PI 3-kinase-derived lipids to the pleckstrin homology domain of PKB allows recruitment of the enzyme to the plasma membrane where it is phosphorylated (and therefore activated) by 3-phosphoinositide-dependent kinase 1, an enzyme that may also require the presence of PI 3-kinase products. We have determined whether leptin stimulation of K\textsubscript{ATP} channel activity in CRI-G1 cells is correlated with any change in PKB isoforms (PKB\textsubscript{α}, -β, and -γ) activities compared with insulin as a positive control. The control activity of PKB isoforms, in the absence of hormones, was 0.87 ± 0.2, 0.24 ± 0.05, and 1.98 ± 0.35 milliunit mg\textsuperscript{-1} protein (n = 3) for PKB\textsubscript{α}, -β, and -γ, respectively. Fig. 5A shows that insulin (10 min exposure) induced a clear activation of PKB\textsubscript{α} and PKB\textsubscript{γ} isoforms with little effect on PKB\textsubscript{β} activity, and the increase in PKB\textsubscript{α} and PKB\textsubscript{γ} isoform activity was inhibited by a 10-min pre-exposure of the cells to 100 nM tolbutamide (n = 3). In contrast, 10 nM leptin (cells stimulated for 5 and 60 min) produced no consistent alteration in PKB isoform activity (n = 3; Fig. 5B). These results are consistent with the PtdIns(3,4,5)P\textsubscript{3} measurements following challenge of CRI-G1 cells with these hormones. Previously, we have shown (23) that insulin stimulation of CRI-G1 cells results in the occlusion of leptin-induced activation of K\textsubscript{ATP} channels indicating some cross-talk between signaling systems. However, application of 10 nM leptin (5–30 min) prior to insulin challenge (Fig. 5C) or 100 nM insulin (10 min) prior to leptin challenge (data not shown) had no significant effect on insulin-stimulated PKB\textsubscript{α} and PKB\textsubscript{γ} isoform activity.

Insulin has also been reported to stimulate mitogenesis and protein synthesis through activation of a 70-kDa ribosomal serine/threonine kinase, p70\textsuperscript{S6K} (13). This insulin-stimulated p70\textsuperscript{S6K} activity appears to be mediated via a PI 3-kinase-dependent pathway, which can be specifically attenuated by the immunosuppressant rapamycin (39, 40). CRI-G1 cells were dialyzed with an electrode solution containing 5 mM ATP and 50 nM rapamycin (Fig. 6, A and B), and the mean resting membrane potential and slope conductance after 10–15 min dialysis were −38.2 ± 1.9 mV and 0.46 ± 0.04 nS, respectively (n = 3). Subsequent application of leptin (10 nM) resulted in hyperpolarization to −73.0 ± 7.3 mV with an increased slope conductance to 3.76 ± 1.00 nS (n = 3) and reversal potential of −79.1 ± 1.7 mV (n = 3) indicating an increase in K\textsuperscript{+} conductance. Tolbutamide (100 μM) completely reversed the leptin induced hyperpolarization (to −42.2 ± 2.3 mV) and increase in slope conductance (to 0.51 ± 0.07 nS) to pre-leptin levels (n = 3).

These data indicate that activation of PKB or p70\textsuperscript{S6K} is
unlikely to underlie leptin activation of $K_{\text{ATP}}$ channels in this cell line. Although PtdIns(3,4,5)P$_3$ itself can activate $K_{\text{ATP}}$ channels, it is plausible that the PI 3-kinase-dependent actions of leptin are not mediated by the synthesis of this lipid, but by the protein serine/threonine kinase activity associated with type I PI 3-kinases. Indeed, Wymann and colleagues (41) re-

**FIG. 3.** PtdIns(3,4,5)P$_3$ activates $K_{\text{ATP}}$ channels in a LY 294002-independent manner. A, a representative current clamp trace of an individual CRI-G1 cell dialyzed with 5 mM ATP and 20 $\mu$M PtdIns(3,4,5)P$_3$. Dialysis with PtdIns(3,4,5)P$_3$ resulted in hyperpolarization of the cell to $-70$ mV, an action that was readily reversed by tolbutamide (100 $\mu$M). B, a plot of the current voltage relations for the currents obtained in A: $\bigcirc$, control; $\bullet$, PtdIns(3,4,5)P$_3$; and $\blacktriangle$, PtdIns(3,4,5)P$_3$ and tolbutamide. PtdIns(3,4,5)P$_3$ increased the membrane conductance relative to control and tolbutamide reversed the PtdIns(3,4,5)P$_3$-induced increase in $K^+$ conductance with a reversal potential of $-75$ mV. C, current clamp trace of a cell dialyzed with 5 mM ATP and 20 $\mu$M PtdIns(3,4,5)P$_3$. Incubation of cells with LY 294002 (10 $\mu$M) for at least 20 min prior to obtaining the whole cell configuration did not prevent the PtdIns(3,4,5)P$_3$-induced hyperpolarization. D, plot of the current voltage relations for the currents obtained in C: LY 294002 ($\bigcirc$), LY 294002 and PtdIns(3,4,5)P$_3$ ($\bullet$), and tolbutamide ($\blacktriangle$). E, representative single channel currents recorded from an inside-out membrane patch exposed to symmetrical 140 mM KCl at a membrane potential of $-40$ mV. Application of 0.1 mM Mg-ATP markedly reduced channel activity. Addition of 1 $\mu$M PtdIns(3,4,5)P$_3$ in the presence of Mg-ATP resulted in a substantial increase in $K_{\text{ATP}}$ channel activity. F, identical experiment to E, using a separate patch that was exposed to 10 $\mu$M LY 294002. Note that the addition of 1 $\mu$M PtdIns(3,4,5)P$_3$ in the presence of LY 294002 does induce a large increase in $K_{\text{ATP}}$ channel activity.
Cells were exposed to 100 nM insulin or 10 nM leptin for CRI-G1 cells, indicating that PD 98059 had no effect (10 nM) hyperpolarized the cells to 2
membrane potential and slope conductance of 0.57 ± 0.07 nS (n = 6), indicating that PD 98059 had no effect per se on $K_{ATP}$ currents. Application of leptin (10 nM) after a 10–15-min dialysis hyperpolarized to −67.2 ± 3.2 mV (n = 6) and increased the slope conductance to 2.86 ± 1.63 nS with an associated reversal potential of −79.0 ± 0.86 mV (n = 6). Tolbutamide (100 μM) completely reversed these actions of leptin (Fig. 6, C and D), such that the membrane potential and slope conductance values returned to −40.2 ± 1.8 mV and 0.53 ± 0.07 nS, respectively (n = 6), values not significantly (p > 0.05) different from control. Thus blockade of the MAPK pathway does not occlude leptin activation of $K_{ATP}$ channels in CRI-G1 insulinoma cells.

Cellular stresses (e.g. UV irradiation and hydrogen peroxide) and certain cytokines have been demonstrated to signal via two MAPK subfamilies, stress-activated MAPK and p38 MAPK, pathways partly controlled by PI 3-kinase (45, 46). Consequently, the effects of SB 203580, a p38 MAPK (47), and at higher concentrations (3–10 μM) an in vitro stress-activated protein kinase (48) inhibitor were examined on the leptin response. CRI G1 cells dialedyzed with an electrode solution containing 5 mM ATP and 10 μM SB 203580 had a mean resting membrane potential and slope conductance of −41.0 ± 3.2 mV and 0.72 ± 0.12 nS (n = 3), respectively. Application of leptin (10 nM) hyperpolarized the cells to −74 ± 4.8 mV and increased the slope conductance to 6.85 ± 2.30 nS (n = 3; data not shown) with a reversal of −79.0 ± 1.4 mV (n = 3) indicating an increase in $K^+$ conductance. Tolbutamide (100 μM) reversed the actions of leptin resulting in membrane potential and conductance values of −42.0 ± 4.3 mV and 0.70 ± 0.07 nS, respectively (n = 3). Consequently, these studies indicate that the common signaling pathways, associated with insulin and growth factor stimulation of cells and that utilize PI 3-kinase as a signaling intermediate, appear not to be functionally connected to the leptin-induced increase in $K_{ATP}$ channel activity in this cell line.

**DISCUSSION**

Inositol phospholipids, applied directly to the cytoplasmic aspect of membrane patches, have been shown to activate native and cloned $K_{ATP}$ channels (29–32). Specifically, PtdIns(4,5)P$_2$ has been proposed as a physiological activator of $K_{ATP}$ channels that functions by disconnecting the channel...
from the constant inhibition produced by the high cytoplasmic concentration of ATP (31, 32). A suggested mechanism to explain this phenomenon is that PtdIns(4,5)P2 binds directly to the C terminus of Kir6.2 and, by charge neutralization, stabilizes the open state of the channel by reducing the inhibitory effect of ATP (31). The present study also demonstrates that PtdIns(4,5)P2 can activate K\textsubscript{ATP} channels of the CRI-G1 cell line when presented directly to the cytoplasmic aspect in the presence of ATP. Surprisingly, however, this effect, like that of leptin, was blocked by inhibitors of PI 3-kinase and was mimicked by the direct application of PtdIns(3,4,5)P3, a key product of type I PI 3-kinases. By contrast, an inhibitor of phosphoinositide-specific PLCs did not prevent the effect of PtdIns(4,5)P2 on K\textsubscript{ATP} channel activation. Taken together these results suggest that channel activation results not from a direct action of PtdIns(4,5)P2 but via a product of its metabolism by a PI 3-kinase, most likely PtdIns(3,4,5)P3.

Recently, it has been shown that the ob gene product leptin activates K\textsubscript{ATP} channels in pancreatic beta cells (8) and CRI-G1 insulin-secreting cells (9), an effect consistent with suppression of insulin secretion. This action of leptin was shown to be blocked by inhibitors of PI 3-kinase (23). These observations, together with the present results, suggested that leptin may exert its effects on K\textsubscript{ATP} channels by stimulating a type I PI 3-kinase to synthesize PtdIns(3,4,5)P3. If correct it would be expected that leptin should produce a detectable increase in the level of PtdIns(3,4,5)P3 present in CRI-G1 cells. Whereas insulin stimulated the production of PtdIns(3,4,5)P3, leptin was without apparent effect using our assay system. One possible explanation for this paradox is that leptin does activate PI 3-kinase to a limited degree, but the resulting increase in PtdIns(3,4,5)P3 may be too small to detect reliably using a measure of the total cell PtdIns(3,4,5)P3 content. Recent studies have shown the existence of a considerable receptor reserve for the PI 3-kinase-dependent activation of PKB by insulin such that substantial activation of PKB occurs at concentrations of insulin that cause barely detectable changes in the total cell PtdIns(3,4,5)P3 content.2 Thus the activity state of PKB, and perhaps other downstream targets of PI 3-kinase signaling pathways, may provide a more sensitive indicator of small changes in PtdIns(3,4,5)P3 content.2

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**FIG. 6.** Rapamycin and PD 98059 do not prevent leptin activation of K\textsubscript{ATP} conductance. A, a representative current clamp trace of a cell dialyzed with 5 mM ATP and 50 μM PD 98059. Dialysis with PD 98059 for 10–15 min had no effect on the resting membrane potential. Addition of leptin (10 nM) after exposure to PD 98059 resulted in hyperpolarization of CRI-G1 cells to −78 mV. Tolbutamide (100 μM) reversed the leptin-induced hyperpolarization to control levels. B, plot of the current-voltage relations for the voltage clamped currents obtained at the times specified in A: PD 98059 (▲), PD 98059 and leptin (●), and tolbutamide (▲). PD 98059 did not prevent leptin-induced increase in K\textsuperscript{+} conductance. C, current clamp record of a separate cell dialyzed with 5 mM ATP and 50 nM rapamycin. Dialysis of cells with rapamycin for at least 10 min had no effect on the resting membrane potential and did not prevent the leptin-induced hyperpolarization. D, plot of current-voltage relationships for the voltage clamped currents obtained in C: rapamycin (▲), rapamycin and leptin (●), and tolbutamide (▲). Rapamycin failed to prevent the leptin-induced increase in K\textsuperscript{+} conductance.

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2 I. H. Batty and C. P. Downes, unpublished data.
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is not required for this leptin-mediated response.

There is clear evidence of cross-talk between insulin and leptin receptor-signaling pathways. For example, leptin attenuates some insulin-induced signals, including gluconeogenesis in human hepatocytes, even though both insulin and leptin stimulate PI 3-kinase in these cells (49). In addition, exposure of CRI-G1 cells to insulin prevents leptin activation of K_ATP channels (23). It was therefore possible that leptin also activates an inhibitory pathway that attenuates some aspects of insulin signaling.

The sensitivity of K_ATP channel activation by leptin to low concentrations of wortmannin and LY294002, however, strongly suggests that a PI 3-kinase is involved in this response. PI 3-kinases have long been known to exhibit both lipid and protein kinase activities that are functions of the same, wortmannin-sensitive active site. A recent study, which made use of a p110 mutant that retained protein kinase activity in the absence of detectable lipid kinase activity, suggested that the protein kinase activity of PI 3-kinase γ mediates the activation of MAPK (41). Furthermore, leptin activates MAPK in Chinese hamster ovary cells stably expressing leptin receptor isoforms (26), a mouse embryonic cell line (24), and the pancreatic beta cell line MIN6 (25). Well characterized inhibitors of the extracellular signal-regulated kinase pathway and pathways involving the related MAPK isoforms, p38 and stress-activated protein kinase, however, all failed to prevent the activation of K_ATP channels by leptin. This suggests that the protein kinase activity of PI 3-kinase does not account for the activation of K_ATP channels by leptin via any of these pathways.

Another possible explanation of our results is that leptin receptor activation may regulate a dedicated pool of PI 3-kinase leading to a localized increase in PtdIns(3,4,5)P_3 that has little effect on the total PtdIns(3,4,5)P_3 content of the cells. In this scenario, PtdIns(3,4,5)P_3 could bind directly to K_ATP channel subunits as proposed originally for PtdIns(4,5)P_2 (31, 32), subsequently lowering ATP sensitivity and causing channel activation. Alternatively, a localized pool of PtdIns(3,4,5)P_3 could act indirectly via its binding to protein targets other than those addressed in this report. Whatever the identity of the putative effector in the pathway for K_ATP channel activation it must display a high degree of selectivity for PtdIns(3,4,5)P_3 over other phosphoinositides, because PtdIns(4,5)P_2 was ineffective (14, 50).

An important feature of lipid signals is that they are likely to remain for some time in the membrane in which they are synthesized. The possibility that PtdIns(3,4,5)P_3 might be generated in different subcellular compartments or membrane microdomains in response to different stimuli may help to explain why signaling by both insulin and leptin can be blocked by PI 3-kinase inhibitors yet these hormones accomplish quite different signaling outcomes. Insulin receptors are not thought to recruit PI 3-kinase directly but instead phosphorylate cytosolic insulin receptor substrate-proteins which in turn bind and activate PI 3-kinases via phosphotyrosine/Src homology 2 domain interactions. Although the leptin receptor, like many other cytokine receptors, is generally considered to signal via janus-tyrosine kinase 2 to various signal transducers and activators of transcription (2), there is evidence that some cytokine receptors (e.g. p55 tumor necrosis factor and epidermal growth factor receptors) can associate directly with phosphoinositide kinases (52). Perhaps leptin receptors can also recruit PI 3-kinases directly leading to a spatially limited production of PtdIns(3,4,5)P_3 and the selective activation of K_ATP channels.

One final possibility to be considered is that the putative PI 3-kinase product elicited by leptin action that is responsible for K_ATP channel activation is not PtdIns(3,4,5)P_3 itself but some other 3-phosphoinositide. Although this will ultimately require direct measurement of these lipids in radiolabeled cells, it is unlikely that phosphatidylinositol 3,4-bisphosphate accounts for these effects, because it is an effective activator of PKB (51, 52), which was not significantly stimulated by leptin in CRI-G1 cells. Other possibilities include PtdIns3P, a product of type II and type III PI 3-kinases, and phosphatidylinositol 3,5-bisphosphate, which is produced by the concomitant phosphorylation of PtdIns by a 3-kinase and the resulting phosphatidylinositol 3-phosphate by a 5-kinase (53). These latter lipids are implicated in vesicle trafficking events and their production is sensitive to nM concentrations of wortmannin.

In conclusion, our results show that activation of K_ATP channels by leptin likely requires PI 3-kinase activity. In support of such a mechanism, PtdIns(3,4,5)P_3 mimicked the effect of leptin by increasing K_ATP channel activity in whole-cell and inside-out current recordings, whereas the effects of PtdIns(4,5)P_2 in these systems were blocked by inhibitors of PI 3-kinase. Surprisingly, however, leptin did not increase the total PtdIns(3,4,5)P_3 content nor did it activate any of several components of established PI 3-kinase signaling pathways. It is possible that either a localized pool of PtdIns(3,4,5)P_3 or some other 3-phosphoinositide might account for these observations. Cell labeling studies, which might allow the detection of alternative lipid mediators, and the use of intracellular probes to monitor the subcellular distribution of PtdIns(3,4,5)P_3 should help to resolve these issues in our future work.

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Essential Role of Phosphoinositide 3-Kinase in Leptin-induced ATP Channel Activation in the Rat CRI-G1 Insulinoma Cell Line
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