Leptin Regulates $K_{\text{ATP}}$ Channel Trafficking in Pancreatic $\beta$-Cells by a Signaling Mechanism Involving AMP-activated Protein Kinase (AMPK) and cAMP-dependent Protein Kinase (PKA)*

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Pancreatic $\beta$-cells secrete insulin in response to metabolic and hormonal signals to maintain glucose homeostasis. Insulin secretion is under the control of ATP-sensitive potassium ($K_{\text{ATP}}$) channels that play key roles in setting $\beta$-cell membrane potential. Leptin, a hormone secreted by adipocytes, inhibits insulin secretion by increasing $K_{\text{ATP}}$ channel conductance in $\beta$-cells. We investigated the mechanism by which leptin increases $K_{\text{ATP}}$ channel conductance. We show that leptin causes a transient increase in surface expression of $K_{\text{ATP}}$ channels without affecting channel gating properties. This increase results primarily from increased channel trafficking to the plasma membrane rather than reduced endocytosis of surface channels. The effect of leptin on $K_{\text{ATP}}$ channels is dependent on the protein kinases AMP-activated protein kinase (AMPK) and PKA. Activation of AMPK or PKA mimics and inhibition of AMPK or PKA abrogates the effect of leptin. Leptin activates AMPK directly by increasing AMPK phosphorylation at threonine 172. Activation of PKA leads to increased channel surface expression even in the presence of AMPK inhibitors, suggesting AMPK lies upstream of PKA in the leptin signaling pathway. Leptin signaling also leads to F-actin depolymerization. Stabilization of F-actin pharmacologically occludes, whereas destabilization of F-actin simulates, the effect of leptin on $K_{\text{ATP}}$ channel trafficking, indicating that leptin-induced actin reorganization underlies enhanced channel trafficking to the plasma membrane. Our study uncovers the signaling and cellular mechanism by which leptin regulates $K_{\text{ATP}}$ channel trafficking to modulate $\beta$-cell function and insulin secretion.

Leptin, a peptide hormone predominantly secreted by white adipocytes, regulates energy homeostasis according to fat storage in the body. It is well established that leptin acts on leptin receptors in hypothalamic neurons to reduce appetite, and disruption of leptin signaling results in obesity. There is growing evidence that, independent of its action on the central nervous system, leptin also has a potent effect on glucose homeostasis by directly targeting pancreatic $\beta$-cells, which secrete insulin in response to elevated plasma glucose levels to promote glucose utilization and storage (1). It has been proposed that leptin and insulin form a dual hormonal feedback loop, termed the adipoinisular axis, to coordinate metabolic control (2). In this axis, insulin secreted from $\beta$-cells is adiopogenic and stimulates leptin release; elevated leptin levels in turn suppress insulin secretion to limit a further increase in fat mass. Supporting this idea, $\beta$-cells express leptin receptors, and leptin reduces insulin secretion in isolated islets and intact animals (3). Dysregulation of the adipoinisular axis results in hyperleptinemia and hyperinsulinemia, leading to the eventual development of type 2 diabetes as seen in animals with $\beta$-cell deletion of leptin receptors (1, 4, 5).

Control of insulin secretion is critically dependent on ATP-sensitive potassium channels ($K_{\text{ATP}}$)3 present in the $\beta$-cell membrane (6–8). The $\beta$-cell $K_{\text{ATP}}$ channel is an octameric complex of four pore-forming inwardly rectifying potassium channel Kir6.2 subunits and four sulfonylurea receptor 1 (SUR1) regulatory subunits (9–11). ATP inhibits channel activity by interacting with Kir6.2, whereas Mg-ATP/ADP stimulates channel activity by interacting with SUR1. As the intracellular ATP/ADP ratio is determined by glucose metabolism, this allows the channel to serve as a metabolic sensor and link glucose metabolism to insulin secretion.

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3 The abbreviations used are: $K_{\text{ATP}}$ channel, ATP-sensitive potassium channel; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; SUR1, sulfonylurea receptor 1; Kir6.2, inwardly rectifying potassium channel Kir6.2; ANOVA, analysis of variance; MNSA, sodium 2-mercaptoethane sulfonate; CC, compound C; BTX, bungarotoxin; PKI, protein kinase A inhibitor; PIP3, phosphatidylinositol 3,4-biphosphate; EGFP, enhanced GFP; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; K-INT, internal potassium solution.

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cose metabolism to membrane potential. Accordingly, when blood glucose levels are low, $K_{ATP}$ channels are open, keeping β-cell membrane potential hyperpolarized to prevent insulin secretion. Glucose stimulation prompts closure of $K_{ATP}$ channels, resulting in membrane depolarization, activation of voltage-gated Ca$^{2+}$ channels, and insulin release. In addition to gating regulation by intracellular nucleotides, $K_{ATP}$ conductance is also a function of channel abundance in the plasma membrane. An increase of channel density will increase the overall channel conductance and thus the threshold of glucose concentrations necessary to depolarize β-cell membrane and stimulate insulin secretion; by contrast, a decrease of channel density is expected to render membrane potential more easily depolarized at a given stimulatory glucose concentration (8). Compared with gating regulation, relatively little is known about how the number of $K_{ATP}$ channels in the β-cell membrane is governed.

That leptin increases $K_{ATP}$ conductance in β-cells was first documented more than 15 years ago (12, 13), and this effect has been proposed to underlie the inhibitory action of leptin on insulin secretion. Several studies have been published reporting the involvement of various signaling molecules and events (14–19); however, the precise mechanism by which leptin increases $K_{ATP}$ channel conductance remains poorly understood. In this study, we show that leptin increases $K_{ATP}$ channel conductance by recruiting channels to the plasma membrane. This regulation is mediated by a signaling mechanism involving the AMP-activated protein kinase (AMPK) and the cAMP-dependent protein kinase (PKA). We demonstrate that leptin signaling leads to F-actin depolymerization, which promotes channel trafficking to the plasma membrane. Our study identifies a physiological signaling mechanism that regulates $K_{ATP}$ channel density at the β-cell membrane to control insulin secretion.

**MATERIALS AND METHODS**

**Molecular Biology**—Rat Kir6.2 cDNA is in the pCDNAl/Amp vector. A minimal α-BTX-binding (WRYYESLEYPD) peptide tag was placed at the N terminus of SUR1 (BTX tag-SUR1) in pECE. The BTX-tag SUR1 and Kir6.2 recombinant adenoviruses were constructed using a modified pShuttle plasmid (AdEasy kit, Stratagene) containing a tetracycline-inducible promoter as described previously (20). Recombinant viruses were amplified in HEK293 cells and purified according to the manufacturer's instructions.

**Cell Culture and Viral Infection**—INS-1 cells (clone 832/13) were cultured in RPMI 1640 medium with 11.1 mM d-glucose (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol (21). Cells at ~70% confluency were washed once with phosphate-buffered saline (PBS) and then incubated for 1.5 h at 37 °C in OptiMEM and a mixture of viruses, including tetracycline-inhibited transactivator and a tetracycline-inhibited transactivator-regulated construct expressing BTX-SUR1 and Kir6.2. The multiplicity of infection for each virus was determined empirically. After 90 min, 2X growth medium was added, and the cells were incubated at 37 °C until reaching appropriate density for the various experiments. Transduction with adenoviruses carrying dominant-negative (AMPKα2-K45R) or constitutively active (AMPKγ1-H150R) AMPK subunits was performed as described previously (22).

**Drug Treatments**—All drugs in this section were purchased from Sigma. For stimulation with leptin, AICAR, forskolin, or 8-bromo-cAMP, INS-1 cells grown in 6-well plates were exposed to regular RPMI 1640 medium without serum for 30 min before treatment with leptin, AICAR, forskolin, or 8-bromo-cAMP for the indicated time or 30 min. Pharmacological inhibitors, including the AMPK inhibitor compound C (CC) or the PKA inhibitors, H89 or protein kinase A inhibitor fragment 14–22 (PKI), were added 30 min before leptin, AICAR, forskolin, or 8-bromo-cAMP treatment.

**Electrophysiology**—For measuring channel sensitivity to ATP and MgADP, the inside-out patch clamp recording configuration was used. Micropipettes had resistance typically ~1–2 megohms. The bath (intracellular) and pipette (extracellular) solutions were K-INT. ATP was added as the potassium salt. The recording was performed at room temperature, and currents were measured at a membrane potential of ~50 mV, and inward currents were shown as upward deflections. Whole-cell patch clamp recording was used to measure $K_{ATP}$ current density in INS-1 cells and β-cells dispersed from human islets (obtained through the Integrated Islets Distribution Program). To identify β-cells, dissociated human islet cells plated on coverslips were placed into the recording chamber and stained briefly (3–5 min) with 10 μg/ml dithizone solution (in PBS) followed by a few minutes of washout with Tyrode’s solution. Previous studies have shown that at this low concentration and short incubation time, dithizone has no deleterious effect on β-cell function (23). Only dithizone-positive cells were recorded. Cells were held at −70 mV, and $K_{ATP}$ currents were recorded at two voltage steps (~50 and ~90 mV) applied every 2 s. Micropipettes were pulled from nonheparinized Kimble glass (Fisher) on a horizontal puller (Sutter Instrument, Novato, CA) and had typical resistance of 3–5 megohms when filled with K-INT solution (140 mM KCl, 10 mM K-HEPES, 1 mM K-EGTA, pH 7.3). Outside Tyrode’s solution contained the following (in mM): NaCl, 137; KCl, 5.4; CaCl$_2$, 1.8; MgCl$_2$, 0.5; Na-HEPES, 5; NH$_4$CO$_3$, 3; Na$_2$HPO$_4$, 0.16; pH 7.2. Diazoxide (200 μM) was applied to the bath solution immediately after break-in to maximally stimulate $K_{ATP}$ channels. After the current had plateaued, 300 μM tolbutamide (a $K_{ATP}$ channel antagonist) was applied to ensure the specificity of the $K_{ATP}$ currents.

**Surface Biotinylation**—INS-1 cells were washed twice with cold PBS. Biotinylation of surface protein was carried out by incubating cells with 1 mg/ml of the membrane-impermeant, thiol-cleavable, amine-reactive biotinylation reagent, EZ-Link Sulfo-NHS-SS-Biotin (Pierce) in PBS for 30 min on ice. The reaction was terminated by incubating cells for 5 min with PBS containing 20 mM glycine, followed by three washes with cold PBS. Cells were then lysed in 300 μl of lysis buffer (50 mM Tris-HCl, 2 mM EDTA, 2 mM EGTA, 100 mM NaCl, 1% Triton X-100, pH 7.4, with complete protease inhibitor) for 30 min at 4 °C. Cell lysate was cleared by centrifugation at 21,000 × g for 10 min at 4 °C, and 500 μg of total lysate was incubated with 100 μl of ~50% slurry of NeutrAvidin-agarose (Pierce) overnight at...
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4 °C. Biotinylated proteins were eluted with 2× protein loading buffer for 15 min at room temperature. Both eluent and input samples (50 μg of total cell lysate) were analyzed by immunoblotting using anti-SUR1 or anti-Kir6.2 antibodies as described previously (24, 25).

To monitor internalization of surface $K_{\text{ATP}}$ channels (Fig. 5A), cells were subjected to surface biotinylation as described above and chased in RPMI 1640 medium containing vehicle or leptin for 15 or 30 min at 37 °C to allow internalization of biotinylated cell surface proteins. At the end of each chase, cells were treated with the membrane-impermeable reducing agent MENSA (50 mM in PBS) for 20 min at 4 °C to strip off the biotin label from proteins remaining at the cell surface. Internalized biotinylated SUR1 protein was then precipitated with NeutrAvidin-agarose beads and analyzed by immunoblotting.

For detecting channels trafficked to the plasma membrane during leptin treatment (Figs. 5B and 6B), INS-1 cells transduced with the BTX-tag SUR1 and Kir6.2 viruses were preincubated with 10 μg/ml unlabeled BTX (Molecular Probes) at 4 °C for 60 min, washed briefly, and then incubated in regular RPMI containing leptin, 8-bromo-cAMP, or AICAR in the absence or presence of PKA or AMPK inhibitors at 37 °C for 30 min. After 30 min, cells were washed with cold PBS, placed on ice, and incubated with 1 μg/ml BTX-conjugated biotin (BTX-biotin; Molecular Probes) for 1 h at 4 °C. Surface BTX-SUR1 labeled with BTX-biotin was then pulled down with NeutrAvidin beads and detected by immunoblotting.

**Immunoblotting**—INS-1 cells were washed twice with ice-cold PBS and lysed in the lysis buffer described above at 4 °C with rotation for 30 min. Cell lysate was cleared by centrifugation at 21,000 × g for 10 min at 4 °C. Small aliquots of the lysates were used for protein determination by the Lowry method (Pierce) with bovine serum albumin as the standard. Proteins were separated by SDS-PAGE (7.5–12.5%) and transferred onto Tris-buffered saline plus 0.1% Tween 20 (TBST). The antibody against phosphoacetyl-CoA carboxylase at Ser-3 and phospho-AMPK at Thr-172 (1:1000 dilutions) was from Millipore, Bedford, MA. Membranes were incubated overnight at 4 °C with a primary antibody diluted in the Tris-buffered saline plus 0.1% Tween 20 (TBST). The antibodies against SUR1 and Kir6.2 (1:500 dilutions) were made as described previously (24). The antibody against phosphoacetyl-CoA carboxylase at Ser-3 and phospho-AMPK at Thr-172 (1:1000 dilutions) was from Millipore. The antibody against IGF-1Rβ (1:1000) was from Santa Cruz Biotechnology, and α-AMPK (1:1000) was purchased from Cell Signaling. After three 10-min washes in TBST buffer, blots were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies in TBST buffer as follows: 1:40,000 goat anti-rabbit IgG (GE Healthcare) for SUR1 and Kir6.2; 1:2000 goat anti-rabbit IgG for phospho-AMPK at Thr-172; 1:2000 horse anti-mouse IgG (GE Healthcare) for total AMPK. Finally, the blots were washed three times for 10 min in TBST and developed using the enhanced chemiluminescence detection kit (Super Signal West Femto, Pierce). The signals were imaged by AlphaView® (Cell Biosciences). Blots were stripped and re-probed with anti-tubulin as a loading control. The blots were quantified with ImageJ (National Institutes of Health) and normalized to the corresponding controls.

**Fluorescence Microscopy**—To visualize surface BTX-tag SUR1, INS-1 cells were infected with the BTX-tag SUR1 and Kir6.2 recombinant adenoviruses as described above and plated onto 18-mm, number 1.5 glass coverslips (Warner Instruments) 24 h post-infection. In images shown in Fig. 2D, cells were treated with leptin or vehicle control for 30 min and then surface BTX-tag SUR1-labeled by incubation with 1 μg/ml Alexa Fluor®555 α-bungarotoxin (Alexa 555-BTX; Molecular Probes) for 1 h at 4 °C. To visualize channels inserted into the plasma membrane during drug treatments (Figs. 5B and 6A), pre-existing surface BTX-tag SUR1 was blocked with unlabeled BTX as described above, and new BTX-tag SUR1 inserted into the membrane following various drug treatments was subsequently labeled with 1 μg/ml Alexa 555-BTX for 1 h at 4 °C. BTX florescence was imaged by confocal microscopy as described below.

For F-actin staining, INS-1 cells were fixed in 4% paraformaldehyde for 30 min. Then cells were washed in PBS and permeabilized in PBS, 0.5% Triton X-100 for 10 min, rinsed in PBS, blocked with 20% normal goat serum (Vector Laboratories) for 30 min, rinsed in PBS, and incubated with 2 units/ml Alexa 488-phalloidin (Invitrogen) for 90–120 min. All imaging experiments were performed on a Zeiss LSM710 three-channel spectral confocal microscope with a 63× 1.4 numerical aperture (NA) objective (Carl Zeiss) under identical conditions with randomly selected regions of each coverslip.

**Statistical Analysis**—All data were analyzed with the program GraphPad Prism™. Results were expressed as mean ± S.E. Differences were tested using one-way analysis of variance (ANOVA) followed by the post hoc Dunnett’s test for multiple comparisons. When only two groups were compared, unpaired Student’s t test was used. The level of statistical significance was set at $p < 0.05$.

**RESULTS**

**Leptin Increases Surface $K_{\text{ATP}}$ Channel Expression**—Leptin has been shown to increase $K_{\text{ATP}}$ channel conductance in β-cells using cell-attached and whole-cell electrophysiological recordings (12, 13). However, it is not known whether this increase results from an effect on channel gating property or channel density. To address this question, we examined the effects of leptin using the rat insulinoma cells INS-1 that express endogenous $K_{\text{ATP}}$ channels and leptin receptors (26, 27). INS-1 cells were treated with 10 nM leptin for 15 or 30 min, conditions previously shown to increase $K_{\text{ATP}}$ conductance. Channel sensitivity to ATP and MgADP, two key physiological ligands that determine channel activity, were assessed by inside-out patch clamp recording. Leptin treatment did not alter channel sensitivity to ATP or MgADP, indicating that the increased conductance is unlikely due to altered channel gating (Fig. 1).

Next, we tested whether leptin affects $K_{\text{ATP}}$ channel abundance at the cell surface. Using surface protein biotinylation as described under “Materials and Methods,” a transient increase in surface SUR1 was observed in cells treated with 10 nM leptin for 15–180 min (Fig. 2A). At the peak effect of 30 min, surface SUR1 was ~4-fold higher than that seen at time 0. Although Kir6.2 does not have extracellular lysine residues that could be biotinylated, it was co-purified with biotinylated SUR1 such that a corresponding increase in surface Kir6.2 was also
observed. Note that because SUR1 is the subunit that is directly labeled by surface biotinylation, surface-biotinylated SUR1 was used to assess surface expression of K\textsubscript{ATP} channels in all subsequent experiments. Interestingly, Western blots of total SUR1 and Kir6.2 proteins showed no difference between control and leptin-treated groups (Student’s t test) were observed under all solution conditions. Error bars are S.E.

To confirm the effect of leptin in primary \( \beta \)-cells, we used whole-cell patch clamp recording to measure K\textsubscript{ATP} current density in dissociated human \( \beta \)-cells treated with leptin or vehicle control. As shown in Fig. 2B, a clear increase in diazoxide-stimulated, tolbutamide-blocked currents characteristic of K\textsubscript{ATP} channels was observed in leptin (10 nM)-treated cells compared with vehicle-treated cells. The averaged current density increase by leptin was 2.67-fold. A similar increase in K\textsubscript{ATP} compared with vehicle-treated cells. The averaged current density increase by leptin was 2.67-fold. A similar increase in K\textsubscript{ATP} channels was observed in leptin (10 nM)-treated cells stimulated, tolbutamide-blocked currents characteristic of K\textsubscript{ATP} channels.

Minimal bungarotoxin-binding motif (BTX tag-SUR1; see under “Materials and Methods”) (28, 29) and performed surface immunostaining using Alexa 555-BTX (red). Nuclei were stained with DAPI (blue). Scale bar, 10 \( \mu \)m. D, INS-1 cells were treated with 10 nM leptin for 15 or 30 min followed by surface biotinylation of membrane proteins. Biotinylated proteins were pulled down with the NeutrAvidin beads and blotted for IGF-1R\( \beta \) or SUR1. Total IGF-1R\( \beta \) and SUR1 present in the whole cell lysate are also shown for comparison.

To directly visualize K\textsubscript{ATP} channels in the cell membrane, we transduced INS-1 cells with recombinant adenoviruses for Kir6.2 and a SUR1 tagged at the extracellular N terminus with a minimal bungarotoxin-binding motif (BTX tag-SUR1; see under “Materials and Methods”) (28, 29) and performed surface immunostaining using Alexa 555-BTX. As shown in Fig. 2C, there is a marked increase in the staining of BTX-tag SUR1 in leptin-treated cells compared with control cells, consistent with an increase in surface channel expression. This result also shows that like endogenous K\textsubscript{ATP} channels, the exogenously expressed channels are subjected to leptin regulation.

To test if the effect of leptin is specific to K\textsubscript{ATP} channels, we examined surface expression of another membrane protein, the insulin growth factor receptor 1 (IGF-1R), after 30 min of 10 nM leptin treatment. No difference in the abundance of surface-biotinylated IGF-1R\( \beta \) was seen between control and leptin-treated cells (Fig. 2D), indicating that leptin does not change global protein expression or membrane trafficking.
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**FIGURE 3.** Leptin increases surface expression of \( K_{ATP} \) channels via activation of AMPK and PKA. **A,** upper panel, Western blots of AMPK phosphorylated at Thr-172 (pAMPK) and total AMPK in INS-1 cells treated with 10 nM leptin for the indicated time. Lower panel, quantification of pAMPK from blots like those shown in the upper panel. The pAMPK signal was normalized to the total AMPK signal and expressed as fold increase of the value seen at time 0. Each bar represents the mean ± S.E. of four separate experiments. \((n = 4/\text{group}; *, p < 0.05 \text{ compared with time 0 by one-way ANOVA and Dunnett's post hoc test.})\) **B,** surface-biotinylated and total SUR1 in INS-1 cells pretreated with DMSO or 10 \( \mu M \) of the AMPK inhibitor compound C (CC) for 30 min before being treated with vehicle (Veh), 10 nM leptin (Lep), or 250 \( \mu M \) of the AMPK activator AICAR (AIC) for another 30 min. C, INS-1 cells were infected with control adenovirus carrying EGFP (Ad-EGFP) or adenovirus carrying a dominant-negative AMPK subunit (Ad-AMPK-DN) to reduce AMPK activity (multiplicity of infection (MOI) of 50 for 48 h). Cells were then treated with vehicle, leptin, or AICAR for 30 min and analyzed for surface and total SUR1. Cell lysate was also blotted for phosphoacetyl-CoA carboxylase to monitor AMPK activity and for tubulin that serves as a loading control. **C,** Ad-EGFP or Ad-AMPK-DN was without effect. **D,** Transduction of INS-1 cells with AICAR (250 \( \mu M \)), an AMPK activator, for 30 min increased surface expression of AMPK. **E,** INS-1 cells were pretreated with DMSO, or the PKA inhibitor H89 (10 \( \mu M \)), or PKI (1 \( \mu M \)) for 30 min before adding vehicle or 10 nM leptin for another 30 min. Surface SUR1 and total SUR1 in the lysate were analyzed as described in the text. Both PKA inhibitors H89 and PKI attenuated the effect of leptin on surface SUR1. **F,** Stimulation of PKA by either forskolin (Fsk; 10 \( \mu M \)) or 8-bromo-cAMP (8-Br; 10 \( \mu M \)) for 30 min was sufficient to mimic the effect of leptin and increase surface SUR1 expression. As controls, co-administration of the PKA inhibitors H89 or PKI with forskolin or 8-bromo-cAMP abrogated the ability of forskolin or 8-bromo-cAMP to increase surface SUR1.

**Effect of Leptin on Surface Expression of \( K_{ATP} \) Channels Is AMPK- and PKA-dependent**—Leptin is known to activate a number of signaling pathways, including AMPK (30, 31). We found that in INS-1 cells, leptin transiently increased phosphorylation of AMPK at threonine 172 with a time course that corresponds to the increase in surface SUR1 (Fig. 3A). To determine whether the effect of leptin on channel expression is dependent on AMPK, we manipulated AMPK activity and tested the effect of leptin. As shown in Fig. 3B, treatment of INS-1 cells with AICAR (250 \( \mu M \)), an AMPK activator, for 30 min increased surface expression of AMPK, mimicking the effect of leptin. Conversely, treatment with an AMPK inhibitor, compound C, precluded the effect of leptin. Moreover, we transduced INS-1 cells with adenoviruses carrying a constitutively active AMPK subunit (AMPK\( \alpha1-H150R \)) or a dominant-negative AMPK subunit (AMPK\( \alpha2-K45R \)) to increase or reduce AMPK activity (22, 32), respectively, as confirmed by an increase or a decrease in the phosphorylation of Ser-79 in acetyl-CoA carboxylase, a downstream substrate of AMPK (Fig. 3, C and D) (30). Manipulations of AMPK activity by expressing the dominant-negative or constitutively active AMPK similarly occluded or mimicked the effect of leptin and AICAR on \( K_{ATP} \) channel surface expression (Fig. 3, C and D), whereas transduction of INS-1 cells with a control EGFP adenovirus (Ad-EGFP) was without effect. Taken together, the above results provide compelling evidence that the effect of leptin on \( K_{ATP} \) channels is mediated by AMPK.

A previous study has reported that \( K_{ATP} \) channel surface expression can be up-regulated by high glucose exposure in a PKA-dependent manner (33). We sought to determine whether PKA has a role in mediating the effect of leptin on \( K_{ATP} \) channel surface expression. INS-1 cells preincubated with the PKA inhibitors H89 (10 \( \mu M \)) or PKI (1 \( \mu M \)) for 30 min before 10 nM leptin treatment failed to show increased \( K_{ATP} \) channel surface expression upon leptin treatment, as judged by the abundance of surface-biotinylated SUR1 (Fig. 3E). On the contrary, activation of PKA by treating cells with 10 \( \mu M \) forskolin or 8-bromo-cAMP for 30 min led to an increase in surface SUR1 (Fig. 3F), resembling that observed in cells treated with leptin. These results indicate that activation of both AMPK and PKA is required for leptin to exert its effect on \( K_{ATP} \) channel density in the plasma membrane.

**AMPK Acts Upstream of PKA in the Leptin Signaling Cascade to Regulate Surface Expression of \( K_{ATP} \) Channels**—Having demonstrated that both AMPK and PKA play a role in mediating the
Leptin Increases Surface K<sub>ATP</sub> Channel Density by Promoting Channel Trafficking to the Plasma Membrane—The density of a membrane protein at the cell surface is a balance between its delivery to and removal from the plasma membrane. We asked whether the increased surface K<sub>ATP</sub> channel expression upon leptin treatment is a consequence of reduced channel endocytosis or increased channel insertion into the plasma membrane. To test the former possibility, a pulse-chase protocol of surface-biotinylated SUR1 was employed. Surface SUR1 was biotinylated at 4 °C and chased for 15 or 30 min at 37 °C in the presence or absence of leptin. At the end of the chase, cells were treated with a reducing agent (MENSA; see under “Materials and Methods”) at 4 °C to remove the biotin label remaining at the cell surface. Internalized biotinylated SUR1 was then affinity-purified and detected by immunoblotting. At 15 min of chase, the amount of internalized biotinylated SUR1 in vehicle and leptin-treated cells was not significantly different (32.51 ± 1.41% versus 28.19 ± 0.79% of total biotinylated SUR1 at time 0). At 30 min, leptin-treated cells showed slightly reduced intracellular biotinylated SUR1 compared with control cells (15 ± 1.64% versus 21.91 ± 1.65% of total biotinylated SUR1 at time 0, p < 0.01, n = 6) (Fig. 5A). In parallel experiments where the residual surface biotin label was not stripped off at the end of each chase, there was no difference in total residual biotinylated SUR1 between leptin-treated and control cells (93.2 ± 6.83% for leptin versus 88.67 ± 11.23% for control, p > 0.05, n = 3), indicating leptin did not alter the degradation rate of surface K<sub>ATP</sub> channels. These results suggest that reduced endocytic trafficking of K<sub>ATP</sub> channels may contribute to the increased surface channel density observed in leptin-treated cells; however, the small decrease in the amount of endocytosed channels is insufficient to account for the increase in surface K<sub>ATP</sub> channels engendered by leptin treatment (~3–4-fold; Fig. 2, A and B).

We next examined whether leptin promotes channel delivery to the cell surface. To monitor channel delivery to the plasma membrane, we again took advantage of the BTX-tag SUR1/Kir6.2 variant, which we have shown to be subjected to leptin regulation like endogenous channels (Fig. 2C). INS-1 cells were transduced with BTX-tag SUR1 and Kir6.2 recombinant adenoviruses. Pre-existing surface BTX-tag SUR1 was first saturated with unlabeled BTX at 4 °C, and cells were then returned to 37 °C in a medium with or without leptin for 30 min. At the end of the incubation, newly inserted channels were labeled with either biotin-conjugated BTX at 4 °C and detected by immunoprecipitation followed by immunoblotting or with Alexa 555-conjugated BTX and detected by fluorescence
microscopy. In both cases, a marked increase in newly inserted channels was observed in leptin-treated cells compared with control cells (Fig. 5B). Quantification of immunoblotting results showed that leptin induced a >2-fold increase in the amount of channels inserted during the 30-min time period (also see bar graph in Fig. 6). These results led us to conclude that leptin increases surface expression of K_ATP channels predominantly by promoting channel trafficking to the plasma membrane.

Leptin Induces F-actin Depolymerization via AMPK/PKA Signaling to Promote K_ATP Channel Trafficking to the Cell Surface—Cytoskeleton remodeling plays a role in membrane trafficking (34, 35). In previous studies, F-actin depolymerization has been found to coincide with leptin-induced increase of K_ATP channel conductance (18). We asked whether actin reorganization has a direct role in leptin-induced channel trafficking to the cell surface. Leptin treatment resulted in reduced F-actin staining by Alexa 488-conjugated phalloidin, indicating F-actin depolymerization (Fig. 6A). As the effect of leptin on surface K_ATP channel abundance is AMPK- and PKA-dependent, we examined whether the reduced F-actin staining observed upon leptin treatment shares the same signaling requirements. As shown in Fig. 6A, activation of AMPK by AICAR or PKA by 8-bromo-cAMP also led to reduced F-actin staining, whereas blocking AMPK with compound C or inhibition of PKA with PKI prevented leptin-induced F-actin remodeling. Consistent with the AMPK and PKA signaling relationship elucidated above, pretreating cells with compound C failed to block PKA-induced F-actin depolymerization. Using the experimental paradigm described above for Fig. 5B, we further determined whether F-actin depolymerization is correlated with increased channel insertion into the plasma membrane. These experiments show that treatments that led to F-actin depolymerization also led to increased channel insertion into the membrane; by contrast, treatments that prevented F-actin depolymerization also failed to yield enhanced channel trafficking to the cell surface (Fig. 6, A and B). These findings revealed a tight correlation between F-actin depolymerization and increased surface insertion of K_ATP channels evoked by the leptin/AMPK/PKA signaling cascade.

To determine whether F-actin depolymerization is required for K_ATP channel trafficking, we treated cells with drugs known to promote or block F-actin depolymerization and monitored cell surface expression of K_ATP channels in response to leptin signaling. In INS-1 cells pretreated for 10 min with 100 nM jasplakinolide, a cyclic peptide that binds and stabilizes filamentous actin (36), leptin failed to increase surface-biotinylated SUR1 (Fig. 7A). The same jasplakinolide treatment also blocked the ability of the AMPK activator AICAR as well as the PKA activators forskolin and 8-bromo-cAMP to increase surface SUR1 (Fig. 7B). These results support the notion that F-actin depolymerization is necessary for leptin, AMPK, and PKA to exert their effects on surface K_ATP channel expression. Conversely, treating cells with the F-actin depolymerizing drug latrunculin B (37) alone without leptin resulted in increased surface expression of K_ATP Channels, indicating that F-actin depolymerization is sufficient to recruit channels to the plasma membrane.

DISCUSSION

As the major determinant of the resting membrane potential of pancreatic β-cells, K_ATP channels serve as gatekeepers to control insulin secretion. In addition to the intricate gating regulation by ATP and ADP in response to glucose metabolism, variation in surface channel density in the plasma membrane is expected to alter total conductance to modulate membrane excitability. The study we present here demonstrates that surface expression of K_ATP Channels is rapidly up-regulated by the hormone leptin. We show that leptin signals through AMPK and PKA to cause actin depolymerization and trafficking of K_ATP channels to the plasma membrane (Fig. 8). The study uncovers a signaling and cellular mechanism by which leptin
regulates $K_{ATP}$ channels and points to $K_{ATP}$ channel trafficking regulation as an important physiological mechanism for controlling channel activity and thus insulin secretion by physiological signals such as leptin.

How leptin increases $\beta$-cell $K_{ATP}$ conductance has been under investigation ever since the phenomenon was first reported more than 15 years ago (12, 13). A number of signaling molecules, including PI3K and tyrosine kinases, were implicated in early studies (14, 16), although their roles were not further substantiated. Subsequent studies show that leptin causes inhibition of the lipid/protein phosphatase PTEN, accumulation of PIP$_3$, and F-actin depolymerization, events that correlate with increased $K_{ATP}$ conductance (15, 18, 19). Despite these efforts, the molecular and cellular events following leptin stimulation that lead to increased $K_{ATP}$ activity remain incompletely understood. Because phosphoinositides phosphatidyl-
inositol 4,5-bisphosphate and PIP3 are known to increase channel open probability and decrease channel sensitivity to ATP inhibition (38, 39), one possibility is that increased channel conductance results from increased PIP3 levels. Analysis of channel sensitivity to ATP inhibition and MgADP stimulation failed to show significant difference between control and leptin-treated cells (Fig. 1), disfavoring an effect of leptin signaling on channel gating property. These results steered us to focus on the alternative that leptin may increase K<sub>ATP</sub> activity by increasing the number of channels present at the cell surface. The biochemical and imaging data we present here provide compelling evidence that leptin increases K<sub>ATP</sub> conductance by promoting channel trafficking to the plasma membrane.

ACTIN REMODELING HAS BEEN SHOWN TO PLAY AN IMPORTANT ROLE IN VESICULAR TRANSPORT ASSOCIATED WITH EXOCYTOSIS (34, 35, 40, 41). IN PANCREATIC β-CELLS, ACTIN DEPOLYMERIZATION PREcedes INSULIN GRANule EXOCYTOSIS IN RESPONSE TO GLUCOSE STIMULATION TO FACILITATE SNARE PROTEIN INTERACTIONS NEEDED FOR MEMBRANE FUSION (42–44). THIS RAISES THE QUESTION OF HOW SECRETORY VESICLES THAT CARRY K<sub>ATP</sub> CHANNELS ARE RELATED TO INSULIN GRANULES. CLEARLY MORE WORK IS NEEDED TO RESOLVE THIS CONTROVERSY.

Leptin has been reported to activate AMPK in a number of cell systems (30, 47, 48). Our data show that leptin leads to increased phosphorylation of AMPK at Thr-172. Several kinases, including LKB1, CaMKK2, and TAK1, can phosphorylate AMPK (49). Although we did not investigate in this study
how leptin leads to increased AMPK phosphorylation, a study just published by Park et al. (50), while this manuscript was under review, reported that leptin activates AMPK via CaMKK2, which is in turn activated by Ca$^{2+}$ influx through TRPC4 channels to promote $K_{ATP}$ channel trafficking to the cell surface. However, the study by Park et al. (50) did not explore the signaling and cellular events following AMPK activation that lead to increased channel trafficking to the cell surface. Interestingly, we found that both PKA inhibitors, H89 and PKI, abolished the ability of leptin and AMPK activators to induce actin depolymerization and $K_{ATP}$ channel recruitment to the plasma membrane; moreover, PKA activators were sufficient to mimic the effect of leptin even in the presence of AMPK inhibitors. These results suggest that PKA acts downstream of AMPK in the leptin signaling pathway. To our knowledge, modulation of PKA activity by AMPK has not been previously documented, although a reverse regulation whereby phosphorylation of AMPK by PKA inhibits AMPK activity has previously been documented, although a reverse regulation whereby phosphorylation of AMPK by PKA inhibits AMPK activity has been reported (51). If PKA is indeed activated by AMPK in the leptin signaling pathway, it would represent a novel downstream target of AMPK. However, until the complete signaling network is mapped out, we cannot exclude the possibility that PKA affects actin depolymerization and $K_{ATP}$ channel trafficking through a parallel pathway that converges with the leptin-AMPK signaling cascade.

The molecular events that link AMPK and PKA activation to actin polymerization and $K_{ATP}$ channel trafficking remain to be determined. Many proteins are involved in actin dynamics and play a role in vesicle trafficking in β-cells, including gelsolin, N-WASP, and coflin (41, 44, 52). Some of these have been shown to be phosphorylated directly by PKA or AMPK, and some are regulated by kinases and phosphatases that are regulated by PKA or AMPK to promote actin depolymerization (53, 54). Actin depolymerization may then allow the mobilization of vesicles previously bound to the F-actin meshwork to permit membrane fusion (40, 44). AMPK and PKA could also phosphorylate $K_{ATP}$ channels directly to affect channel trafficking. It is worth noting that a previous study has identified a PKA phosphorylation site in human SUR1 that appears to be involved in channel surface expression regulation (55). Phosphorylation of $K_{ATP}$ channels by AMPK has also been proposed to modulate channel function (56, 57), although the details remain to be worked out. Whether channel phosphorylation is involved in the effect of leptin needs further investigation. In addition to proteins, phosphoinositides phosphatidylinositol 4,5-bisphosphate and PIP$_3$ are well recognized for their roles in cytoskeleton remodeling and membrane trafficking in β-cells (58) and other cell types in general (59). As leptin signaling has also been reported to increase PIP$_3$ levels (18, 19), an important question to address in the future is whether changes in phosphoinositides account for the observed F-actin depolymerization and $K_{ATP}$ channel trafficking.

Leptin and insulin, secreted by adipocytes and β-cells, respectively, have been proposed to form a negative adipoinselular feedback loop to prevent hyperinsulinemia and hyperleptinemia. It is worth noting that in our experiments, the effect of leptin on AMPK phosphorylation and $K_{ATP}$ channel surface expression peaked at ~30 min and diminished with longer incubation (Fig. 2A). Intriguingly, circulating leptin levels in humans have been reported to follow a pulsatile pattern with ~30 pulses every 24 h and each pulse lasting ~30 min (60). The transient nature indicates that the leptin signaling pathway is tightly regulated. We speculate that the ability to turn off the leptin signal may prevent prolonged inhibition of insulin secretion when a subsequent rise in glucose calls for more insulin release.

In summary, we have uncovered a signaling pathway by which leptin modulates the abundance of $K_{ATP}$ channels in the plasma membrane to regulate insulin secretion. Aside from β-cells, neurons in the central nervous system involved in a wide range of physiological and pathological processes, including energy homeostasis (18, 61–63), epilepsy (64, 65), and Parkinson disease (66), express $K_{ATP}$ channels and are subjected to regulation by leptin and metabolic signals. Thus, the $K_{ATP}$ channel regulatory mechanism identified here may extend well beyond pancreatic β-cells, with broad implications in metabolic regulation in health and disease.

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Addendum—While this manuscript was being considered for publication, Park et al. (50) reported similar findings that leptin increases $K_{ATP}$ channel trafficking to the plasma membrane.

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