Leptin promotes K_{ATP} channel trafficking by AMPK signaling in pancreatic β -cells

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Leptin is a pivotal regulator of energy and glucose homeostasis, and defects in leptin signaling result in obesity and diabetes. The ATP-sensitive potassium (K_{ATP}) channels couple glucose metabolism to insulin secretion in pancreatic β -cells. In this study, we provide evidence that leptin modulates pancreatic β -cell functions by promoting K_{ATP} channel translocation to the plasma membrane via AMP-activated protein kinase (AMPK) signaling. K_{ATP} channels were localized mostly to intracellular compartments of pancreatic β -cells in the fed state and translocated to the plasma membrane in the fasted state. This process was defective in leptin-deficient *ob/ob* mice, but restored by leptin treatment. We discovered that the molecular mechanism of leptin-induced AMPK activation involves canonical transient receptor potential 4 and calcium/calmodulin-dependent protein kinase kinase β . AMPK activation was dependent on both leptin and glucose concentrations, so at optimal concentrations of leptin, AMPK was activated sufficiently to induce K_{ATP} channel trafficking and hyperpolarization of pancreatic β -cells in a physiological range of fasting glucose levels. There was a close correlation between phospho-AMPK levels and β -cell membrane potentials, suggesting that AMPK-dependent K_{ATP} channel trafficking is a key mechanism for regulating β -cell membrane potentials. Our results present a signaling pathway whereby leptin regulates glucose homeostasis by modulating β -cell excitability.

The K_{ATP} channel, an inwardly rectifying K⁺ channel that consists of pore-forming Kir6.2 and regulatory sulfonylurea receptor 1 (SUR1) subunits (1), functions as an energy sensor: its gating is regulated mainly by the intracellular concentrations of ATP and ADP. In pancreatic β -cells, K_{ATP} channels are inhibited or activated in response to the rise or fall in blood glucose levels, leading to changes in membrane excitability and insulin secretion (2, 3). Thus, K_{ATP} channel gating has been considered an important mechanism in coupling blood glucose levels to insulin secretion. Recently, trafficking of K_{ATP} channels to the plasma membrane was highlighted as another important mechanism for regulating K_{ATP} channel activity (4–6).

AMP-activated protein kinase (AMPK) is a key enzyme regulating energy homeostasis (7). We recently demonstrated that K_{ATP} channels are recruited to the plasma membrane in glucose-deprived conditions via AMPK signaling in pancreatic β -cells (6). Inhibition of AMPK signaling significantly reduces K_{ATP} currents, even after complete wash-out of intracellular ATP (6). Given these results, we proposed a model that recruitment of K_{ATP} channels to the plasma membrane via AMPK signaling is crucial for K_{ATP} channel activation in low-glucose conditions. However, the physiological relevance of this model remains unclear because pancreatic β -cells had to be incubated in media containing less than 3 mM glucose to recruit a sufficient number of K_{ATP} channels to the plasma membrane (6). We thus hypothesized that there should be an endogenous ligand in vivo that promotes AMPK-dependent K_{ATP} channel trafficking sufficiently to stabilize pancreatic β -cells at physiological fasting glucose levels.

Leptin is an adipocyte-derived hormone that regulates food intake, body weight, and glucose homeostasis (8, 9). In addition

to its central action, leptin regulates the release of insulin and glucagon, the key hormones regulating glucose homeostasis, by direct actions on β - and α -cells of pancreatic islets, respectively (10–12). It thus was proposed that the adipoinular axis is crucial for maintaining nutrient balance and that dysregulation of this axis contributes to obesity and diabetes (12). However, intracellular signaling mechanisms underlying leptin effects are largely unknown. Leptin was shown to increase K_{ATP} currents in pancreatic β -cells (13, 14), but the possibility that K_{ATP} channel trafficking mediates leptin-induced K_{ATP} channel activation has not been explored.

In the present study, we demonstrate that the surface levels of K_{ATP} channels increase in pancreatic β -cells under fasting conditions in vivo. Translocation of K_{ATP} channels to the plasma membrane in fasting was absent in pancreatic β -cells from *ob/ob* mice, but restored by treatment with leptin, suggesting a role for leptin in K_{ATP} channel trafficking in vivo. We further show that leptin-induced AMPK activation, which is essential for K_{ATP} channel trafficking to the plasma membrane, is mediated by activation of canonical transient receptor potential 4 (TRPC4) and calcium/calmodulin-dependent protein kinase kinase β (CaMKK β). Our results highlight the importance of trafficking regulation in K_{ATP} channel activation and provide insights into the action of leptin on glucose homeostasis.

Results

Leptin Induces K_{ATP} Channel Trafficking to the Plasma Membrane. We previously demonstrated that K_{ATP} channels translocate to the plasma membrane of pancreatic β -cells under low-glucose conditions via AMPK signaling (6). To investigate whether K_{ATP} channel trafficking occurs in vivo depending on feeding status (fasted vs. fed), we isolated and immediately fixed pancreatic tissues from wild-type (WT) mice either at 1 h after feeding (WT fed) or after a 12-h fasting period (WT fasted). We compared the distribution of K_{ATP} channels in the β -cells of pancreatic islets using specific antibodies against SUR1 and Kir6.2 (Fig. 1*A* and *B* and Fig. S1). In the pancreas from WT fed mice, SUR1 and Kir6.2 were localized mostly to intracellular compartments and uniformly distributed throughout the cytoplasm of islet cells. In WT fasted mice, a distinctive staining pattern representing the translocation of the K_{ATP} channel toward the cell periphery was observed in the islet cells (Fig. 1*A*). These findings confirm that K_{ATP} channel trafficking is physiologically regulated in vivo by feeding status.

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In our previous *in vitro* study using the insulin-secreting cell line INS-1, glucose concentration less than 3 mM was required to induce maximal AMPK activation and K_{ATP} channel trafficking (6). However, the mean blood glucose level in the WT fed mice was 244 ± 14 mg/dL (~ 13.5 mM, $n = 10$), and that in the WT fasted mice was 138 ± 11 mg/dL (~ 7.7 mM, $n = 10$), which may not be sufficient to fully activate AMPK activity. Therefore, we supposed the presence of an endogenous ligand *in vivo* that induces AMPK activation and K_{ATP} channel trafficking and tested the idea that leptin plays this role using *ob/ob* mice lacking this hormone. In contrast to observations in WT mice, a distinct staining pattern indicating surface translocation of SUR1 and Kir6.2 was lost in islet cells of *ob/ob* mice obtained after a 12-h fasting period (Fig. 1*B* and Fig. S1). Interestingly, this pattern was restored when *ob/ob* mice were treated with leptin for 4 d (2 μ g/d) (Fig. 1*B* and Fig. S1), indicating that leptin is critical for the surface translocation of Kir6.2 during fasting *in vivo*.

Intracellular localization of K_{ATP} channels has been studied by several groups, but results are controversial (4, 16). Because endosomal recycling is important for regulation of the density of surface proteins (17), we tested the colocalization of K_{ATP} channels with early endosomal antigen 1 (EEA1), an endosomal marker. The results show significant colocalization of Kir6.2 with EEA1 (Fig. 1*A*, Lower and Fig. S1*B*). Interestingly, EEA1 also is translocated toward the cell periphery and colocalized almost completely with Kir6.2 in β -cells in the islets of WT fasted and leptin-treated fasted *ob/ob* mice (Fig. 1*A* and *B*, Lower and Fig. S1*B*).

To confirm whether regulation of K_{ATP} channel trafficking by feeding status has functional significance, we measured whole-cell K^+ currents in β -cells in pancreatic slices obtained from fed and fasted mice. To mimic the difference in glucose concentrations depending on feeding status *in vivo*, slices obtained from fed mice were superfused with 17 mM glucose, and those from fasted mice were superfused with 6 mM glucose. To maximize K_{ATP} channel open probability and to minimize channel rundown, we used ATP- and Mg^{2+} -free internal solutions (6, 18). According to the previous report (19), we identified β -cells in slices when ATP wash-out caused an immediate increase in K_{ATP} currents (Fig. 1*C*). The maximum whole-cell conductance measured after complete wash-out of intracellular ATP was normalized to the cell capacitance (6.3 pF, $n = 15$), and this value (G_{max}) was regarded to represent K_{ATP} conductance (details in

SI Materials and Methods). G_{max} in β -cells in pancreatic slices obtained from fasted mice was 3.97 ± 0.48 nS/pF ($n = 8$), which was significantly larger than that from the fed mice (1.41 ± 0.22 nS/pF, $n = 6$) (Fig. 1*C*). Provided that the open probability of K_{ATP} channels reaches the maximum under the above experimental conditions, the difference in G_{max} depending on feeding status likely is attributable to the difference in surface density of K_{ATP} channels. We also tested the K_{ATP} channel distribution pattern and G_{max} in isolated pancreatic β -cells from rats and INS-1 cells. Kir6.2 was localized mostly in the cytosolic compartment in isolated β -cells and INS-1 cells cultured in media containing 11 mM glucose without leptin, but translocated to the cell periphery when cells were treated with leptin (10 nM) for 30 min (Fig. 1*D*). Consistent with this finding, leptin treatment increased G_{max} significantly in both β -cells [from 1.62 ± 0.37 nS/pF ($n = 12$) to 4.97 ± 0.88 nS/pF ($n = 12$); Fig. 1*E*] and INS-1 cells [from 0.9 ± 0.21 nS/pF ($n = 12$) to 4.1 ± 0.37 nS/pF ($n = 10$) in leptin; Fig. 1*E*]. We confirmed that the leptin-induced increase in G_{max} was reversed by tolbutamide (100 μ M), a selective K_{ATP} channel inhibitor (Fig. S2).

AMPK Mediates Leptin-Induced K_{ATP} Channel Trafficking. To investigate molecular mechanisms of leptin action on K_{ATP} channels trafficking, we performed *in vitro* experiments using INS-1 cells that were cultured in the media containing 11 mM glucose. We measured surface levels of Kir6.2 before and after treatment of leptin using surface biotinylation and Western blot analysis. Unless otherwise specified, cells were treated with leptin or other agents at room temperature in normal Tyrode's solution containing 11 mM glucose. We also confirmed key results at 37 °C (Fig. S3). The surface levels of Kir6.2 increased significantly following treatment with 10 nM leptin for 5 min and further increased slightly at 30 min (Fig. 2*A*). Parallel increases in STAT3 phosphorylation levels (Fig. S4*A*) ensured proper leptin signaling under our experimental conditions (20). In contrast, the surface levels of Kir2.1, another inwardly rectifying K^+ channel in pancreatic β -cells, were not affected by leptin (Fig. S4*B*). Because the total expression levels of Kir6.2 were not affected by leptin (Fig. 2*A*), our results indicate that leptin specifically induces translocation of K_{ATP} channels to the plasma membrane.

K_{ATP} channel trafficking at low glucose levels was mediated by AMPK (6). We examined whether AMPK also mediates leptin-

Fig. 1. The effect of fasting on K_{ATP} channel localization *in vivo*. (*A* and *B*) Pancreatic sections were prepared from wild-type (WT) mice at fed or fasted conditions and *ob/ob* mice under fasting conditions without or with leptin treatment. Immunofluorescence analysis used antibody against SUR1. (*A* and *B*, Lower) Immunofluorescence analysis using antibodies against Kir6.2 (green) and EEA1 (red). The images are enlarged from the indicated boxes in Fig. S1*B*. (*C*) Pancreatic slice preparation with a schematic diagram for patch clamp configuration (in blue box) and the voltage clamp pulse protocol. Representative traces show K_{ATP} current activation in single β -cells in pancreatic slices obtained from fed and fasted mice. Slices obtained from fed mice were superfused with 17 mM glucose, and those from fasted mice were superfused with 6 mM glucose. The bar graph shows the mean data for G_{max} in β -cells from fed and fasted mice. The error bars indicate SEM. *** $P < 0.005$. (*D*) Immunofluorescence analysis using anti-Kir6.2 antibody and in rat isolated β -cells and INS-1 cells in the absence [Leptin (-)] and presence [Leptin (+)] of leptin in 11 mM glucose. (*E*) Representative traces for K_{ATP} current activation in INS-1 cells (Left) and the mean data for G_{max} in INS-1 cells and isolated β -cells (Right). Error bars indicate SEM. *** $P < 0.005$.

Fig. 2. Leptin promotes K_{ATP} channel trafficking to the plasma membrane and increases K_{ATP} channel currents via AMPK in INS-1 cells and primary β -cells. (A–D) Cells were treated with leptin in normal Tyrode's solution containing 11 mM glucose for the indicated time period before surface labeling with a biotin probe. (A) Surface (S) and total (T) fractions were probed using the indicated antibodies. AMPK activity was assessed based on the levels of pAMPK and pACC in Fig. S4A. (B) Cells were transfected with the indicated siRNAs for 48 h and then treated with leptin for 30 min before surface biotinylation. scRNA, scrambled siRNA against AMPK; siAMPK, siRNA against AMPK. (C) Cells were incubated with leptin and/or 10 μ M compound C (CC) for 30 min before surface biotinylation. (D) The relative ratios of surface to total Kir6.2, surface to total SUR1, and pAMPK to total AMPK were plotted based on the quantification of the band intensities ($n = 3$ –6). (E) Cells were treated with leptin and/or CC for 30 min before confocal microscopy for assessing subcellular distribution of Kir6.2. (F) The maximum whole-cell conductance (in nanosiemens) was measured when current activation reached steady state and normalized by the cell capacitance (in picofarads) under each experimental condition indicated below the graph ($n = 12$ –20). (G) Variance and mean analysis of the K_{ATP} current in control (black) and leptin-treated cells (red). The bar graph shows the number of cell surface K_{ATP} channels per cell (N/cell). Error bars indicate SEM. * $P < 0.05$, *** $P < 0.005$.

induced K_{ATP} channel trafficking. Western blot analysis showed that phosphorylation levels of AMPK (pAMPK) and its substrate acetyl-CoA carboxylase (pACC) increased following treatment with leptin (Fig. 2A and Fig. S4A). Furthermore, the time course and magnitude of leptin-induced AMPK phosphorylation were matched perfectly with those of leptin-induced K_{ATP} channel trafficking (approximately a threefold increase at 5 min; Fig. S4C).

Next, we performed knockdown experiments using siRNA against AMPK α -subunits (siAMPK), as described in our previous study (6). The siAMPK markedly reduced total and pAMPK in leptin-treated INS-1 cells. Moreover, leptin barely increased Kir6.2 surface levels in siAMPK-transfected cells (Fig. 2B and D). The total expression levels of the K_{ATP} channel were not affected by leptin or transfection of siAMPK or scrambled siRNA (scRNA). Pharmacological inhibition of AMPK with compound C (CC) (21) also inhibited the effect of leptin on the surface level of Kir6.2 (Fig. 2C and D). These results were confirmed further by immunofluorescence analyses. Leptin treatment for 30 min enhanced Kir6.2 signal at the cell periphery, but this leptin effect was significantly inhibited by CC (Fig. 2E). For quantitative analysis, the ratio of peripheral to total Kir6.2 signal was obtained from the line scan data, and the mean values in each condition were shown in the bar graph (Fig. S4D). Consistent with the role of AMPK in leptin-induced K_{ATP} channel trafficking,

leptin-induced increase in G_{max} was inhibited by siAMPK and CC (Fig. 2F). We also confirmed the inhibitory effect of CC on the leptin-induced increase in G_{max} in primary β -cells (Fig. 2F).

To confirm that the leptin-induced increase in G_{max} is indeed attributable to the increase in surface channel number (N), we performed noise analysis. To calculate the N, the variance and mean values of the K_{ATP} currents measured during the removal of intracellular ATP were fitted with parabola function (details in SI Materials and Methods and Fig. S5). The N increased from 438 ± 48 ($n = 11$) to $1,247 \pm 87$ ($n = 15$) by leptin treatment (Fig. 2G), suggesting that ~ 800 K_{ATP} channels translocate to the cell surface by leptin treatment, and the leptin-treated cells have a K_{ATP} channel density approximately three times higher (56.57 ± 6.81 N/pF vs. 152.50 ± 10.44 N/pF) in the plasma membrane.

CaMKK β Mediates Leptin-Induced AMPK Activation. Because CaMKK β and the protein kinase LKB1 are upstream kinases of AMPK (22, 23), we examined which one mediates AMPK activation in leptin-treated INS-1 cells. The siRNA against CaMKK β (siCaMKK β) markedly decreased leptin-induced AMPK phosphorylation, whereas siLKB1 did not affect leptin action on AMPK phosphorylation (Fig. 3A). The CaMKK β inhibitor 7-oxo-7H-benzimidazo[2,1-a]benz[de]isouquinoline-3-carboxylic acid acetate (STO-609) (24) also significantly decreased leptin-induced AMPK phosphorylation, confirming that CaMKK β acts as an upstream kinase of AMPK in leptin signaling (Fig. 3B and Fig. S3). In addition, leptin-induced increases in the Kir6.2 surface level and G_{max} were almost completely abolished by STO-609 (Fig. 3E and Fig. S3). Because CaMKK β is activated in a Ca^{2+} -dependent manner (22), we examined whether Ca^{2+} is critical for leptin-induced AMPK activation. When INS-1 cells were treated with BAPTA-AM (20 μ M), a membrane permeable Ca^{2+} buffering agent, leptin-induced AMPK phosphorylation decreased markedly (Fig. 3C). Together, our findings indicate that leptin activates AMPK by CaMKK β , which leads to K_{ATP} channel trafficking.

Next, we examined whether leptin indeed induces an increase of cytosolic Ca^{2+} using Fura-2 Ca^{2+} imaging. At 11 mM glucose, INS-1 cells showed a variable degree of Ca^{2+} oscillations. Leptin induced a biphasic effect on cytosolic Ca^{2+} concentrations in six of nine cells tested (Fig. S6), and the mean Ca^{2+} concentration obtained from these cells is demonstrated in Fig. 3D. Upon addition of 10 nM leptin, the amplitude and frequency of Ca^{2+} oscillation were increased significantly, followed by almost

Fig. 3. Leptin-induced AMPK activation is mediated by CaMKK β activation in INS-1 cells. (A) Cells were transfected with siLKB1 or siCaMKK β and then treated with 10 nM leptin for 30 min before Western blot analysis ($n = 3$). (B and C) Cells were treated with 10 nM leptin and/or 5 μ M STO-609 or 20 μ M BAPTA-AM before Western blot analysis. (D) Measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in INS-1 cells using Fura-2. The data are expressed as the mean values ($n = 6$). (E) K_{ATP} channel activity was measured using whole-cell patch clamp analysis in the cells treated with 10 nM leptin and/or the indicated agents [5 μ M STO-609, 50 μ M Ni^{2+} , 10 μ M nimodipine (Nimo), 2 μ M thapsigargin (TG), or 100 μ M 2-APB] ($n = 8$ –20). Error bars indicate SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$; ns, not significant.

complete cessation of Ca^{2+} oscillations, possibly as the result of activation of K_{ATP} channels.

Leptin-Induced TRPC4 Activation Underlies AMPK Activation by Leptin. We then investigated the Ca^{2+} transport pathway that mediates leptin-induced $\text{CaMKK}\beta$ activation. Whole-cell patch clamp analysis using pharmacological blockers revealed that the leptin-induced increase in G_{max} was unaffected by the L-type Ca^{2+} channel inhibitor nimodipine (10 μM), the T-type Ca^{2+} channel inhibitor Ni^{2+} (50 μM), or the sarco/endoplasmic reticulum Ca^{2+} -ATPase inhibitor thapsigargin (2 μM) but significantly attenuated by the TRPC channel blocker 2-aminoethyl-diphenyl borate (2-APB) (100 μM) (Fig. 3E). These results suggest that leptin causes Ca^{2+} influx through TRPC channels. Thus, we examined whether TRPC channels are present and regulated by leptin in INS-1 cells.

To identify functional expression of TRPC channels, we characterized nonselective cation conductance while outward K^{+} currents were blocked by a Cs^{+} -based internal solution. Because external Cs^{+} fully activates TRPC current (25), we compared the nonselective cation currents (I_{NSC}) induced by replacing external Na^{+} with Cs^{+} under various conditions (Fig. 4A, Left). Voltage ramp pulses from +100 to -100 mV (0.4 V/s) were applied, and the current-voltage (I-V) relationship for I_{NSC} was obtained by subtracting the I-V relationship in Na^{+} solution from that in Cs^{+} solution. This I-V relationship exhibited a double rectification profile with a negative slope conductance at voltages around -70 mV and the reversal potential around 0 mV (Fig. 4A, Right). These characteristics are known to be typical of TRPC channels (26). When cells were pretreated with leptin for 30 min, we observed a significant increase in the double-rectifying nonselective cation currents. The amplitude of I_{NSC} measured at -70 mV was 50.0 ± 5.0 pA ($n = 10$) in control, and this was increased to 110.0 ± 12.6 pA ($n = 10$) by leptin treatment. Leptin activates TRPC channels via phosphoinositide 3-kinase (PI3K) in the hypothalamus (27). We confirmed that the leptin-induced increase in I_{NSC} was completely abolished in the presence LY294002 (10 μM), a PI3K inhibitor (Fig. 4A).

TRPC4 and TRPC5 are the most likely candidates for receptor-operated Ca^{2+} -permeable nonselective cation channels (28). Therefore, we tested the effect of gene knockdown for endogenous

TRPC4 or TRPC5 from INS-1 cells. In siTRPC4-transfected cells, basal I_{NSC} was significantly reduced compared with those of siGFP- and siTRPC5-transfected cells (Fig. 4B). Furthermore, the leptin-induced increase in I_{NSC} was significantly attenuated in siTRPC4-transfected cells (Fig. 4B), but not in siTRPC5-transfected cells. These results suggest that TRPC4 is the major TRPC subunit that underlies I_{NSC} in INS-1 cells and is activated by leptin signaling.

We also tested whether leptin-induced AMPK activation is specifically mediated by TRPC4. Leptin-induced AMPK phosphorylation was inhibited by siTRPC4 (Fig. 4C and D) and the TRPC4 blocker ML204 (Fig. S2), but not by siTRPC5 (Fig. 4C and D). Finally, we confirmed that the leptin-induced increase in G_{max} was abolished by siTRPC4, but not by siTRPC5 (Fig. 4E). From these results, we concluded that leptin signaling involving PI3K/TRPC4/ $\text{CaMKK}\beta$ leads to the activation of AMPK and K_{ATP} channel trafficking.

Leptin Augments AMPK Activation and Hyperpolarization at Fasting Glucose Levels. To understand the physiological significance of leptin's effect on β -cell excitability, we measured the resting membrane potential (RMP) of INS-1 cells in the physiologically relevant ranges of glucose and leptin concentrations. Serum leptin levels in humans with normal body weight are reported to be 0.5–1 nM (29), and we assumed that 1 nM is close to the physiological concentration of leptin. We treated INS-1 cells with different concentrations of glucose (0, 3, 6, or 11 mM) in normal Tyrode's solution for 2 h, and measured RMP using a perforated patch method to maintain the physiological intracellular milieu. At 11 mM glucose, which is the concentration in culture media, the RMP of INS-1 cells fluctuated, with a mean value of -35.5 ± 1.5 mV ($n = 10$, Fig. 5A, Left). Some cells showed firing of spontaneous action potentials. Application of 1 nM leptin showed little effect on RMP at 11 mM glucose, but 10 nM leptin caused significant hyperpolarization, reaching steady levels in about 10 min (-59.8 ± 1.6 mV, $n = 12$; Fig. 5A). After preincubation of cells with 6 mM glucose, which is close to the fasting blood glucose level, for 2 h, the RMP still remained depolarized (-37.2 ± 1.2 mV, $n = 6$; Fig. 5A, Center), but addition of 1 nM leptin induced significant hyperpolarization (-61.5 ± 1.5 mV, $n = 6$; Fig. 5A, Center), indicating that leptin is crucial to allow sufficient hyperpolarization at fasting glucose levels. Leptin-induced hyperpolarization was reversed rapidly by tolbutamide (Fig. 5A, Center), confirming that the leptin effect on RMP was mediated by activation of the K_{ATP} current. Even in the absence of leptin, glucose deprivation for 2 h induced sufficient hyperpolarization (-65.7 ± 1.5 mV, $n = 10$; Fig. 5B). In the presence of CC, however, the RMP remained depolarized, even at 0 mM glucose (Fig. 5A, Right), and 10 nM leptin failed to induce hyperpolarization (-34.0 ± 1.6 mV, $n = 10$; Fig. 5A, Right). Mean values under each condition plotted in Fig. 5B indicate that hyperpolarization of RMP at low glucose concentrations is mediated by AMPK signaling and this effect is augmented by leptin.

For quantitative analyses of the relationship between AMPK signaling and β -cell RMP, we measured pAMPK levels using microtiter plate assays from the INS-1 cells incubated with different glucose concentrations (0, 6, 11, or 17 mM) in normal Tyrode's solution for 2 h in the absence or presence of 1 nM or 10 nM leptin. Glucose deprivation induced maximal AMPK activation, which was not activated further by leptin. At 6 mM glucose, AMPK was activated slightly in the absence of leptin (black rectangles in Fig. 5C), but markedly activated in the presence of 1 nM leptin (red circles in Fig. 5C). At 11 mM glucose, 1 nM leptin could not, but 10 nM leptin could, induce near maximum AMPK activation (blue triangles in Fig. 5C). These results indicate that AMPK activation at low glucose levels is augmented by leptin in a dose-dependent manner. Using the data shown in Fig. 5B and C, we plotted mean RMP values obtained at each condition vs. corresponding pAMPK levels (Fig. 5D). The linear relationship between RMP and pAMPK

Fig. 4. TRPC4 activation underlies leptin-induced AMPK phosphorylation in INS-1 cells. (A and B) Cells were treated with 10 nM leptin and/or indicated agents (siGFP, siTRPC4, siTRPC5, or 10 μM LY294002) before patch clamp analysis. Leptin-induced I_{NSC} was recorded as described in *SI Materials and Methods*. (C and D) Cells were transfected with siTRPC4 or siTRPC5 and then incubated with 10 nM leptin for 30 min before Western blot analysis. The relative pAMPK-to-total AMPK ratio was plotted based on the quantification of the band intensities ($n = 3-6$). (E) K_{ATP} channel activity in the denoted conditions was measured using whole-cell patch clamp analysis ($n = 10-20$). Error bars indicate SEM. *** $P < 0.005$.

Fig. 5. Effects of glucose and leptin concentrations on resting membrane potentials and AMPK activities. Leptin augments AMPK activation and hyperpolarization at low glucose concentrations in INS-1 cells. (A) Cells were treated with 0, 6, or 11 mM glucose plus 1 or 10 nM leptin. Tolb, tolbutamide; CC, compound C. A perforated patch method was used to assess resting membrane potentials (RMPs). (B and C) The plot represents the relationship between glucose concentrations and RMPs or AMPK activities obtained in the presence of 0, 1, and 10 nM leptin with or without CC. Physiological range of glucose concentration is indicated with gray boxes. Error bars indicate SEM ($n = 6$ –12 for RMP or $n = 3$ for AMPK activity). (D) The plot represents the relationship between AMPK activities and RMP changes. (E) The islets were treated with 8, 13, or 16 mM glucose and/or leptin at 37 °C before Western blot analysis. (F) Schematic diagram for the signaling pathway involved in leptin-induced K_{ATP} channel trafficking.

levels indicates that AMPK is a key regulator for β -cell RMP. Taken together, we concluded that leptin at physiological concentrations facilitates AMPK activation at fasting glucose levels so that K_{ATP} channel trafficking is promoted to hyperpolarize β -cell RMP.

The role of leptin in β -cell response to lowering glucose concentrations was tested further using pancreatic islets isolated acutely from WT and *ob/ob* mice. Isolated islets were incubated in media with different glucose concentrations for 1 h and examined with regard to subcellular localization of Kir6.2 and level of pAMPK. In islets isolated from WT fed mice, Kir6.2 translocation and pAMPK phosphorylation were induced when the glucose concentration in the media was lowered to 8 mM, which is equivalent to the blood glucose level of WT fasted mice, from 13 mM glucose, which is equivalent to the blood glucose level in WT fed mice (Fig. 5E and Fig. S7A). In the islets obtained from *ob/ob* fasted mice, however, Kir6.2 translocation and AMPK activation were not induced at 8 mM glucose and were induced only when leptin (10 nM) was added (Fig. 5E and Fig. S7B). These results indeed suggest that the effect of fasting on K_{ATP} channel trafficking observed in vivo (Fig. 1A) is mediated by AMPK activation by glucose concentration changes within physiological ranges in the presence of leptin.

Discussion

Leptin regulates glucose homeostasis through central and peripheral pathways (12, 30). We now demonstrate that AMPK activation, recruitment of K_{ATP} channels to the cell surface, and the increase in K_{ATP} conductance are induced at fasting glucose concentrations in β -cells in pancreatic islets obtained from WT mice. On the contrary, in β -cells in *ob/ob* mice islets or in culture,

additional leptin is required to induce these changes. Our data not only show the physiological significance of leptin actions, but also provide a mechanism for a direct action of leptin on pancreatic β -cells. Leptin induces AMPK activation in pancreatic β -cells, which leads to an increase in K_{ATP} channel trafficking to the plasma membrane.

Signaling Mechanism for AMPK Activation by Leptin in Pancreatic β -Cells. Involvement of AMPK signaling in leptin effects has been well demonstrated in skeletal muscle and hypothalamus (31), but it remains unclear in pancreatic β -cells (32). In the present study, we elucidated the signaling mechanism for leptin-induced AMPK activation in pancreatic β -cells. CaMKK β , but not LKB1, mediates leptin-induced AMPK activation, and TRPC4 is involved in CaMKK β activation (Figs. 3 and 4). We also demonstrated that leptin induces a rise in intracellular Ca²⁺ concentrations (Fig. 3D). Taken together, it might be concluded that Ca²⁺ signals induced by TRPC4 activation are essential for leptin-induced AMPK activation, which in turn promotes K_{ATP} channel trafficking to the plasma membrane (Fig. 5F).

In the present study, however, we did not directly study the downstream mechanisms linking AMPK activation to K_{ATP} channel translocation, but we showed that EEA1 is colocalized and translocated with K_{ATP} channels by leptin (Fig. 1 *A* and *B* and Fig. S1*B*). Previous reports showed colocalization of K_{ATP} channels with secretory granules containing insulin (16) or chromogranin (4) in cultured pancreatic β -cells. Colocalization of K_{ATP} channels with EEA1 may suggest a possibility that K_{ATP} channels are localized to the endosomal recycling compartment and translocated to the cell surface by AMPK signaling. Considering that endocytic recycling comprises multiple steps that involve complicated molecular mechanisms (17), further studies are required to clarify the molecular mechanisms regulating K_{ATP} channel trafficking by AMPK.

Physiological Significance of Leptin-Induced AMPK Activation in Pancreatic β -Cells. In the present study, we performed quantitative analysis of the effect of leptin on AMPK activation by low glucose levels (Fig. 5). The results imply that leptin signaling facilitates AMPK activation by low glucose levels. Molecular mechanisms involved in this facilitating action of leptin must be determined, but its pathophysiological significance is evident. AMPK may be almost fully activated in the range of fasting glucose levels in the presence of a physiological concentration of leptin. In leptin-deficient conditions, however, AMPK signaling cannot respond sensitively to a low energy status, whereas at high concentrations of leptin, AMPK is activated irrespective of glucose concentrations. Under both conditions, the ability of AMPK to sense energy status is impaired, so the role of AMPK in regulating energy homeostasis may be compromised. The implication of these results is that leptin concentration is important to optimize the sensitivity of AMPK signaling to cellular energy status, so AMPK can be sufficiently activated at fasting glucose levels and inhibited at fed glucose levels.

We further determined the effects of glucose concentrations and leptin on RMPs (Fig. 5B). The results strikingly resemble those of pAMPK levels (Fig. 5C). Given that RMPs have a linear relationship to pAMPK levels (Fig. 5D) and the surface levels of K_{ATP} channels are regulated by pAMPK levels (Fig. 2), we propose a model in which the K_{ATP} channel trafficking mediated by AMPK is the key mechanism for regulating pancreatic β -cell RMPs in response to glucose concentration changes.

It generally is believed that the sensitivity of the pancreatic β -cell's responses to glucose concentration changes depends on the ATP sensitivity of K_{ATP} channel gating (2, 3). At low glucose concentrations, the open probability (P_O) of K_{ATP} channels is enhanced by an increase in MgADP associated with a decrease in ATP. However, at physiologically relevant glucose levels, K_{ATP} channels have very low P_O (33, 34), and the range of P_O change is narrow (in ref. 31, 7% and 3% of maximum P_O in 5 mM and 10 mM glucose, respectively). Thus, it has been

questioned whether gating regulation of K_{ATP} channels by MgADP and ATP is sufficient to induce glucose-dependent membrane potential changes in pancreatic β -cells. We showed that AMPK-dependent K_{ATP} channel trafficking serves as another important mechanism for β -cell membrane potential regulation. We measured Kir6.2 surface density by Western blotting (Fig. 2A–C) and noise analysis (Fig. 2G) and showed that the increase in Kir6.2 surface density by leptin is about threefold, which is no less than the dynamic range of P_O changes by MgADP and ATP.

The role of AMPK in pancreatic β -cell functions also is supported by a recent study using mice lacking AMPK α 2 in their pancreatic β -cells, in which reduced glucose concentrations failed to hyperpolarize pancreatic β -cell membrane potential (35). Interestingly, glucose-stimulated insulin secretion (GSIS) also was impaired by AMPK α 2 knockout (35), suggesting that the maintenance of hyperpolarized membrane potential at low blood glucose levels is a prerequisite for normal GSIS. The study did not consider K_{ATP} channel malfunction in these impairments, but K_{ATP} channel trafficking very likely is impaired in AMPK α 2 in pancreatic β -cells, causing a failure of hyperpolarization at low glucose concentrations. It also is possible that impaired trafficking of K_{ATP} channels affects β -cell response to high glucose stimulation, but this possibility remains to be studied.

We also show the crucial role of leptin on K_{ATP} channel trafficking to the plasma membrane at fasting glucose concentrations in vivo (Fig. 1). These results are in line with our model that leptin is required for maintaining sufficient density of K_{ATP} channels in the β -cell plasma membrane, which guarantees appropriate regulation of membrane potential under resting conditions, acting primarily during fasting to dampen insulin secretion. In this context, hyperinsulinemia associated with leptin deficiency (*ob/ob* mice) or leptin receptor deficiency (*db/db* mice) may be explained by impaired tonic inhibition due to insufficient K_{ATP} channel density at the surface membrane. Because there

is a strong relationship among increased basal insulin levels, obesity, and diabetes in humans (36, 37), a mechanism to dampen insulin secretion during fasting may provide therapeutic strategies for inhibiting development of obesity-related diabetes.

Materials and Methods

We used INS-1 cells (passage 20–50) for electrophysiology, Western blot analysis, and immunocytochemistry experiments. INS-1 cells were cultured on poly-L-lysine-coated coverslips in RPMI-1640 medium containing 10% (vol/vol) FBS and 11 mM D-glucose. Changes in the surface level of K_{ATP} channels were detected by surface biotinylation/streptavidin purification and subsequent Western blot analysis using anti-Kir6.2 antibody (Santa Cruz Biotechnology). Specificity for anti-Kir6.2 was examined using siKir6.2 transfected cells (Fig. S8). AMPK activation was detected by a commercial ELISA kit (Invitrogen) or by Western blot analysis using phosphorylation-specific antibodies to AMPK α at Thr172 (pAMPK) and its substrate, pACC, from Cell Signaling Technology. Full scans of all Western blots indicating regions shown in the respective main figures are shown in Fig. S9.

Immunofluorescence analysis was performed using pancreatic tissue sections and isolated pancreatic islets obtained from female C57BL/6 WT and *ob/ob* mice at age 7–8 wk (Shizuoka, Japan), as well as INS-1 cells. Information about antibodies used in the present study is provided in Tables S1 and S2. All animal experimental procedures were conducted in accordance with the guidelines of the University Committee on Animal Resources at Seoul National University (approval no. SNU-120216-02). Confocal images were obtained using a Fluoview 1000 (Olympus) or TCS-SP2 (Leica) confocal microscope and processed with Leica Confocal Software.

See *SI Materials and Methods* for details on electrophysiological measurements using the patch clamp technique, intracellular $[Ca^{2+}]$ measurement using microfluorimetry with Fura-2-acetoxymethyl ester (AM), composition of experimental solutions, drugs, and statistical analysis.

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