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Leptin inhibits insulin secretion induced by cellular cAMP in a pancreatic B cell line (INS-1 cells)

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Ahrén, Bo, and Peter J. Havel. Leptin inhibits insulin secretion induced by cellular cAMP in a pancreatic B cell line (INS-1 cells). *Am. J. Physiol.* 277 (*Regulatory Integrative Comp. Physiol.* 46): R959–R966, 1999.—The effect of leptin on insulin secretion is controversial due to conflicting results in the literature. In the present study, we incubated insulin-producing rat insulinoma INS-1 cells for 60 min and examined the effects of recombinant murine leptin (20 nmol/l). We found that leptin (0.1–100 nmol/l) did not affect the insulin response to glucose (1–20 mmol/l). However, when cells were incubated with agents that increase the intracellular content of cAMP, i.e., glucagon-like peptide-1 (100 nmol/l), pituitary adenylate cyclase activating polypeptide (100 nmol/l), forskolin (2.5 µmol/l), dibutyryl-cAMP (1 mmol/l), or 3-isobutyl-1-methylxanthine (100 µmol/l), leptin significantly reduced insulin secretion (by 34–58%, $P < 0.05$–0.001). In contrast, when insulin secretion was stimulated by the cholinergic agonist carbachol (100 µmol/l) or the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (1 µmol/l), both of which activate protein kinase C, leptin was without effect. We conclude that leptin inhibits insulin secretion from INS-1 cells under conditions in which intracellular cAMP is increased. This suggests that the cAMP-protein kinase A signal transduction pathway is a target for leptin to inhibit insulin secretion in insulin-producing cells.

glucagon-like peptide-1; pituitary adenylate cyclase activating polypeptide-38; forskolin; adenosine 3′,5′-cyclic monophosphate; 3-isobutyl-1-methylxanthine; carbachol; protein kinase C; calcium

LEPTIN IS EXPRESSED in adipocytes and secreted into the bloodstream in proportion to adipose tissue mass (2, 6, 7, 20) and recent energy intake (3, 11, 22, 54). Although there is increasing evidence that leptin production is regulated by insulin (21, 46), probably via changes of adipocyte glucose metabolism (41), the effects of leptin on insulin secretion are more controversial due to a number of conflicting reports in the literature. Leptin acts through specific leptin receptors, which belong to the class I cytokine receptor family (52). A main site of action of leptin is the hypothalamus, where it has the effect of inhibiting food intake and stimulating energy expenditure (6, 19, 43). However, several studies have shown that leptin also acts peripherally. For example, leptin receptors are present in bone marrow and in the liver (16, 53).

In 1996, Kieffer and collaborators (28) reported that leptin receptors are also expressed in the insulin-producing B cells within the pancreatic islets, suggesting that leptin might influence insulin secretion through a direct action on these cells. This was later confirmed by Emilsson et al. in 1997 (12) as well as by several other investigators (9, 13, 32, 42, 44, 51). It is important to determine the functional role of these receptors in regulating islet function, because leptin levels are increased in obese subjects (2, 6, 7, 20). Obese subjects with reduced insulin sensitivity exhibit a compensatory hypersecretion of insulin and hyperinsulinemia that helps to prevent glucose intolerance (33). However, if hyperleptinemia affects this islet adaptation to reduced insulin sensitivity, leptin could potentially have a role in the development of glucose intolerance in obese subjects.

Leptin has been shown to inhibit insulin secretion from insulin-producing insulinoma cells (15, 32, 56), isolated rodent (9, 12, 24, 29, 32, 42, 44, 48, 56) and human (13, 32, 38) islets, the perfused rodent pancreas (14, 15), and in vivo in mice (32). However, there are also conflicting reports in the literature. Some studies using an isolated perfused rat pancreas model found that leptin did not affect insulin secretion (34–36), whereas in other studies, leptin was shown to stimulate insulin secretion in islets and insulinoma cells (8, 49, 51). Therefore, conflicting data exist regarding the influence of leptin on insulin secretion.

In early 1998, Poitout and collaborators (44) reported that leptin preferentially inhibits insulin secretion in the presence of 3-isobutyl-1-methylxanthine (IBMX), which raises the level of cAMP through a nonspecific inhibition of phosphodiesterase (PDE) subspecies. These results supported the previous findings of a potent inhibitory effect of leptin reported in the presence of the glucoincretin, glucagon-like peptide-1 (GLP-1; 14, 56). Furthermore, a recent study has shown that leptin inhibits insulin secretion by activating the PDE 3B subspecies of PDE (56), which would reduce the content of cAMP, thereby preventing specifically the cAMP-protein kinase A (PKA)-induced insulin secretion. These findings led us to the working hypothesis that leptin preferentially inhibits insulin secretion when cAMP levels are elevated. In this report, we present experimental evidence for this hypothesis in the insulin-producing INS-1 rat insulinoma cell line. This cell line was used because it retains morphologically differentiated characteristics compared with other cell lines and also shows a marked insulinotropic response to glucose and to increases of the cellular content of cAMP induced by forskolin (4).

METHODS

Cells. INS-1 cells [derived from an X-ray-induced insulinoma in the rat exhibiting characteristics resembling the

normal B cell (4); cells were a kind gift from Professor Claes Wollheim, Geneva, Switzerland] were cultured at 37°C in humidified 5% CO_2 in air in RPMI 1640 medium (GIBCO BRL, Paisley, UK) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin (all Kebo Laboratory, Spånga, Sweden), and 50 µM β-mercaptoethanol (GIBCO) (4). Cells were subcultured every 7–10 days by trypsination, and the medium was changed after each subculture and thereafter every fourth day. Cells were used for experiments after reaching confluency at 7–10 days after subculture. The cells were used in experiments in passage *numbers 76–84*.

Insulin secretion experiments. Cells were seeded into 24-well plates (Nunc, Roskilde, Denmark) at a concentration of 0.5 million cells/well and cultured for 48 h. The cells were then washed twice in HEPES medium containing (in mmol/l) 125 NaCl, 5.9 KCl, 1.28 $CaCl_2$, 1.2 $MgCl_2$, 25 HEPES, and 0.1% human serum albumin (pH 7.36; all Sigma Chemical, St. Louis, MO) and incubated in a volume of 200 µl with 1 mmol/l glucose for 30 min. Thereafter, the medium was changed and the cells were incubated in the HEPES medium supplemented with varying concentrations of glucose, recombinant murine leptin (Amgen, Thousand Oaks, CA), synthetic GLP-1, synthetic ovine pituitary adenylate cyclase activating polypeptide-38 (PACAP-38; both Peninsula Europe, Merseyside, UK), forskolin, IBMX, dibutyryl-cAMP, CCK-8, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (all Sigma), and/or carbachol (BDH, Poole, UK) according to the protocols. In one experimental series, leptin (20 nmol/l, which is approximately 10 times higher than circulating levels of leptin; Ref. 2) was also added during the 30-min preincubation. After 60 min, an aliquot of 150 µl was collected from each well and centrifuged at 350 g for 5 min. Aliquots of 50 µl were then stored at 20°C until analysis of insulin by means of a radioimmunoassay using guinea pig anti-porcine insulin, mono-^{125}I-insulin, and, as standard, rat insulin (Linco, St. Charles, MO). Free and bound radioactivity were separated by use of an anti-IgG (goat anti-guinea pig) antibody (Linco).

Measurements of Ca^{2+}. Cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) was determined in fura 2-AM-loaded INS-1 cells according to a previously described protocol (30). In brief, cells were grown for 4–7 days in RPMI medium supplemented as above. After trypsination, cells were recovered for 2 h in 10 ml of RPMI medium supplemented with 10% fetal calf serum at 37°C in 5% CO_2 and were thereafter loaded with fura 2-AM (1 µmol/l; Sigma) for 45 min. After equilibration for 20 min, 2 ml of the cell suspension (0.5 million cells/ml) were transferred to a cuvette for measurement of $[Ca^{2+}]_{cyt}$ in a Perkin-Elmer LS-50 spectrophotofluorometer at 37°C. Forskolin and leptin were added at defined time points and remained in the cuvette until the end of the experiment. Excitation wavelengths were 340 and 380 nm, and the emission wavelength was 510 nm. Fluorescence maximum was obtained by adding 0.03% Triton X, and fluorescence minimum was obtained by adding EGTA in excess at the end of experiments performed in the absence of albumin. $[Ca^{2+}]_{cyt}$ was calculated according to Grynkiewicz et al. (18).

Measurements of cellular cAMP. INS-1 cells were seeded on four-well plates (0.5 million cells/well) and cultured for 48 h. The cells were then washed twice in the HEPES medium and incubated at 37°C in a volume of 200 µl in the presence of 10 mmol/l glucose and 0.1 mmol/l IBMX with or without the addition of forskolin and leptin. The incubation was stopped after 2 min with addition of ice-cold ethanol (final concentration 65%), and the cells were scraped off with a rubber policeman. After being washed twice in 65% ice-cold ethanol, the extracts were centrifuged at 2,000 g at 4°C for 15 min, transferred to fresh test tubes, evaporated at 60°C under a

stream of nitrogen, and then stored at −20°C until analysis for protein content by the Lowry method (37) and for cAMP by radioimmunoassay, using a rabbit antisuccinyl AMP serum, cyclic 2-succinyl-3-^{125}I-methyl ester as tracer, and cAMP as standard (Amersham, Amersham, UK). Free and bound radioactivity were separated by the double antibody technique. The results are reported as amount of cellular cAMP divided by amount of cellular protein.

Statistics. The results are reported as means ± SE. Statistical evaluation of differences between groups was performed by one-way ANOVA followed by Bonferroni post hoc test or by two-tailed Student's t-test for unpaired comparison. $P < 0.05$ was considered significant.

RESULTS

Glucose-stimulated insulin secretion. Figure 1 shows that insulin secretion from INS-1 cells incubated for 60 min was stimulated by glucose in a concentration-dependent manner with a maximal stimulation at 10 mmol/l. The addition of leptin at a concentration of 20 nmol/l, added either during the 60-min incubation only or also during the 30-min preincubation period, had no effect on glucose-stimulated insulin secretion. Furthermore, leptin at concentrations ranging from 0.1 to 100 nmol/l had no influence on the insulin secretory response to glucose at a fixed glucose concentration of 10 mmol/l (Fig. 2). Furthermore, when the cells were incubated in the presence of a nonstimulatory glucose concentration (3 mmol/l) together with leptin at 0.5, 1, 5, 10, 20, or 50 nmol/l, no influence of leptin was observed on insulin secretion (data not shown in Fig. 2). Hence, over a wide range of concentrations, leptin had no effect on glucose-stimulated insulin secretion from INS-1 cells whether administered with glucose during the incubation only or during the preincubation period as well.
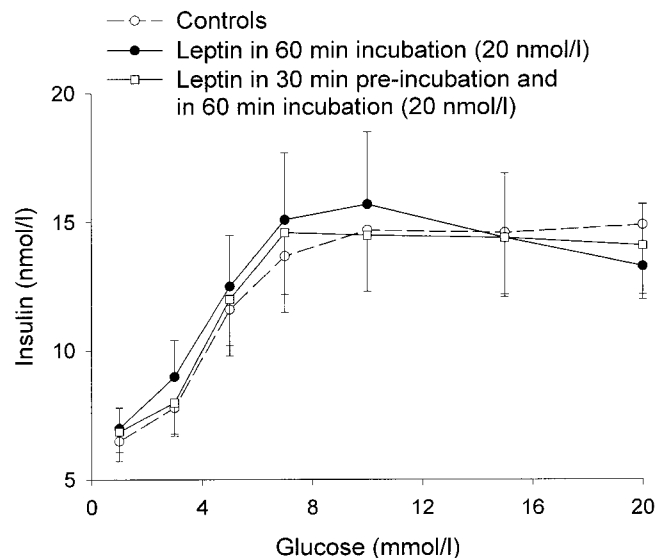

Fig. 1. Insulin secretion from INS-1 cells cultured for 60 min in presence of different concentrations of glucose with or without addition of leptin (20 nmol/l) during incubation period only or during 30-min preincubation period as well. Means ± SE are shown, $n = 24$ wells examined in 4 different experiments.
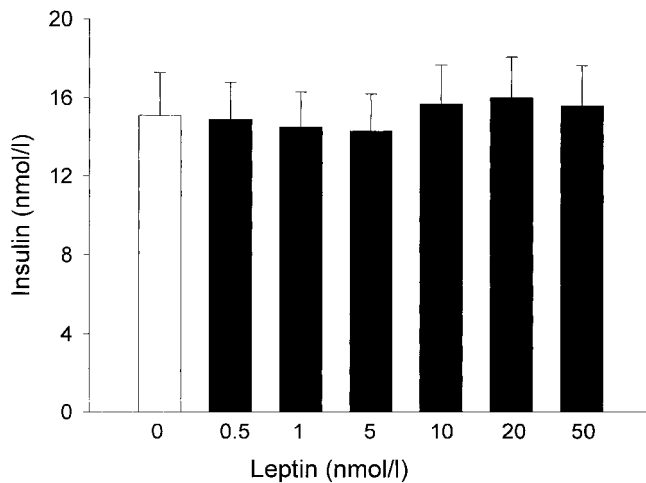
Fig. 2. Insulin secretion from INS-1 cells cultured for 60 min in presence of 10 mmol/l glucose and different concentrations of leptin. Means ± SE are shown, $n = 16$ wells examined in 4 different experiments.

GLP-1- and PACAP-38-induced insulin secretion. Both GLP-1 (100 nmol/l) and PACAP-38 (100 nmol/l) markedly potentiated glucose (10 mmol/l)-stimulated insulin secretion from INS-1 cells (Fig. 3). Leptin (20 nmol/l) significantly inhibited insulin secretion stimulated by GLP-1 by 58% and by PACAP by 46%, suggesting that a signaling pathway activated by these peptide hormones, but not by glucose, is targeted by leptin.

Forskolin- and cAMP-induced insulin secretion. Because the insulinotropic action of both GLP-1 and PACAP-38 has been thought to largely rely on formation of cAMP (1, 10, 17, 30, 50), we examined the influence of leptin (20 nmol/l) on insulin secretion stimulated by forskolin (2.5 µmol/l), which activates

adenylate cyclase, or dibutyryl-cAMP (1 mmol/l), which mimics the effects of endogenously produced cAMP. Both forskolin and dibutyryl-cAMP markedly stimulated insulin secretion in INS-1 cells and leptin inhibited insulin secretion stimulated by these agents by 34 and 35%, respectively (Fig. 4). This result suggests that leptin inhibits insulin secretion under conditions when intracellular cAMP is elevated.

Glucose-stimulated insulin secretion in the presence of IBMX. The nonspecific PDE inhibitor IBMX is known to increase the cellular content of cAMP and thereby potentiate glucose-stimulated insulin secretion by exaggerating the PKA pathway (25, 39). Leptin (20 nmol/l) inhibited insulin secretion induced by IBMX (100 µmol/l) in the presence of 10 mmol/l glucose by 48% (Fig. 4), further suggesting that an increased level of intracellular cAMP is required to detect an insulinostatic effect of leptin in INS-1 cells.

Carbachol-, TPA-, and CCK-8-stimulated insulin secretion. To determine whether the inhibition by leptin of insulin secretion is specific for the cAMP-PKA pathway, we also examined the influence of leptin on insulin secretion in the presence of agents known to target protein kinase C (PKC), i.e., the cholinergic agonist carbachol and the phorbol ester TPA (25). Leptin (20 nmol/l) did not affect carbachol (100 µmol/l)- or TPA (1 µmol/l)-stimulated insulin secretion (Fig. 5). The effects of the COOH-terminal octapeptide of the gut hormone cholecystokinin on insulin secretion were also examined, because this gut hormone is known to stimulate insulin secretion largely through activation of PKC (26). However, we found that CCK-8 did not stimulate insulin secretion in INS-1 cells when incubated over a wide range of doses (0.1 nmol/l–1 µmol/l) at 10 mmol/l glucose (data not shown). Therefore, the combination of CCK-8 and leptin was not examined.

Cytoplasmic Ca^{2+}. In addition to activation of PKA, formation of cAMP is also known to stimulate exocyto-
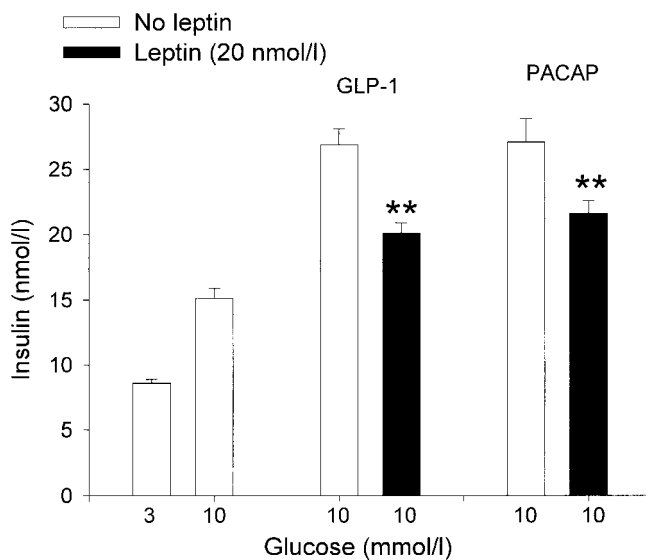

Fig. 3. Insulin secretion from INS-1 cells cultured for 60 min in presence of 3 or 10 mmol/l glucose with or without addition of glucagon-like peptide (GLP)-1 (100 nmol/l) or pituitary adenylate cyclase activating polypeptide (PACAP)-38 (100 nmol/l) and/or leptin (20 nmol/l). Means ± SE are shown, $n = 18$ wells examined in 3 different experiments. Probability level of random difference vs. glucose control: ** $P < 0.01$.
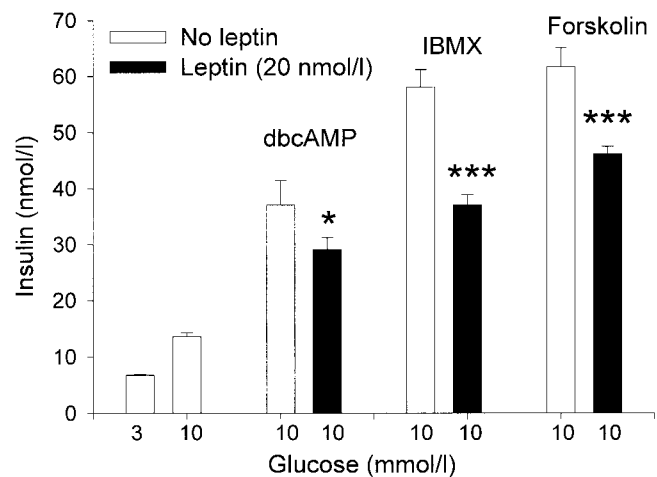

Fig. 4. Insulin secretion from INS-1 cells cultured for 60 min in presence of 3 or 10 mmol/l glucose with or without addition of dibutyryl-cAMP (dbcAMP; 1 mmol/l), 3-isobutyl-1-methylxanthine (IBMX; 100 µmol/l), or forskolin (2.5 µmol/l) and/or leptin (20 nmol/l). Means ± SE are shown, $n = 16$ wells examined in 4 different experiments. Probability level of random difference vs. glucose control: * $P < 0.05$, *** $P < 0.001$.
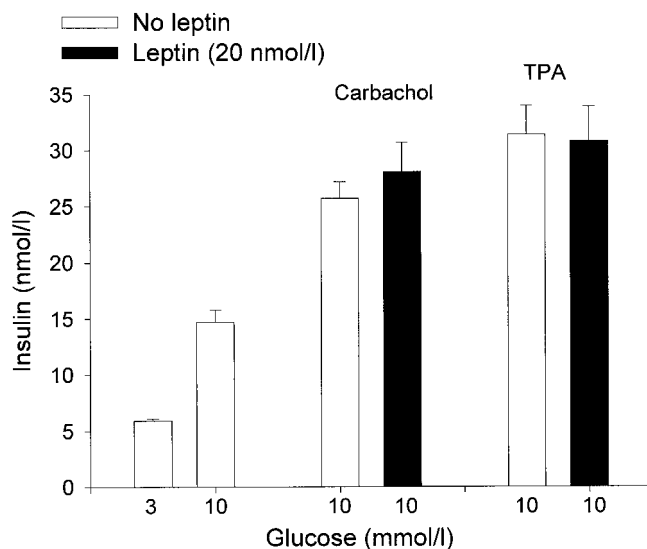
Fig. 5. Insulin secretion from INS-1 cells cultured for 60 min in presence of 3 or 10 mmol/l glucose with or without addition of carbachol (100 μmol/l), 12-*O*-tetradecanoylphorbol 13-acetate (TPA; 1 μmol/l), and/or leptin (20 nmol/l). Means ± SE are shown, $n = 18$ wells examined in 3 different experiments.

sis by increasing $[Ca^{2+}]_{cyt}$ (17). To determine whether leptin inhibits forskolin-induced increase in $[Ca^{2+}]_{cyt}$ in INS-1 cells, fura 2-AM-loaded cells were used. In the presence of 10 mmol/l glucose, forskolin (2.5 μmol/l) increased $[Ca^{2+}]_{cyt}$. However, leptin (20 nmol/l) did not affect either this increase or the baseline $[Ca^{2+}]_{cyt}$ (Fig. 6). Similarly, inclusion of leptin (20 nmol/l) during the 45-min loading period did not affect the subsequent forskolin-induced increase in $[Ca^{2+}]_{cyt}$. Finally, leptin did not affect the increase in $[Ca^{2+}]_{cyt}$ induced by depolarization with KCl (20 mmol/l; data not shown).

Cellular cAMP. To examine whether the inhibition by leptin of insulin secretion stimulated by agents raising cAMP is due to degradation of formed cAMP, INS-1 cells were incubated in the presence of forskolin (2.5 μmol/l) with or without addition of leptin (20 nmol/l) at 10 mmol/l glucose. It was found that the cellular cAMP content in controls incubated at 10 mmol/l glucose without addition of forskolin or leptin was 45.9 ± 4.2 pmol/mg protein. This was raised by addition of forskolin to 86.4 ± 4.3 pmol/mg protein ($P < 0.001$). The cellular content of cAMP after combined addition of forskolin and leptin was 88.5 ± 5.8 pmol/mg protein, which is not significantly different from that after incubation with forskolin alone. Hence, leptin does not affect the increased cellular content of cAMP induced by forskolin, suggesting that under these conditions, leptin does not stimulate the degradation of cAMP.

DISCUSSION

In this study we found that incubation with leptin did not affect glucose-stimulated insulin secretion from rat insulinoma INS-1 cells when examined over a wide range of leptin concentrations and with a range of glucose levels. In contrast, leptin inhibited insulin secretion induced by agents that increase the intracellular levels of cAMP. Therefore, the present study

provides novel evidence that leptin specifically inhibits insulin secretion when cellular cAMP is elevated using a variety of interventions that activate or potentiate the PKA signal transduction pathway.

To exclude the possibility that the failure of leptin to inhibit the insulinotropic response to glucose was due to a short incubation period (see Ref. 45), in one series of experiments leptin was included in the 30-min preincubation period as well, but again no effect on insulin secretion was observed. Moreover, we did not detect any influence of leptin on $[Ca^{2+}]_{cyt}$ in the presence of 10 mmol/l glucose or on the cellular content of cAMP in the presence of glucose and forskolin. These results suggest that although leptin receptors are expressed in INS-1 cells (15), leptin does not interact with the signaling pathways regulating the insulinotropic action of glucose in this cell line. Previous studies have shown a weak inhibitory influence of leptin on insulin secretion in HIT-T15 cells (leptin 2 nmol/l; glucose 11 mmol/l; 56), in βTC-6 cells (leptin 10 nmol/l;
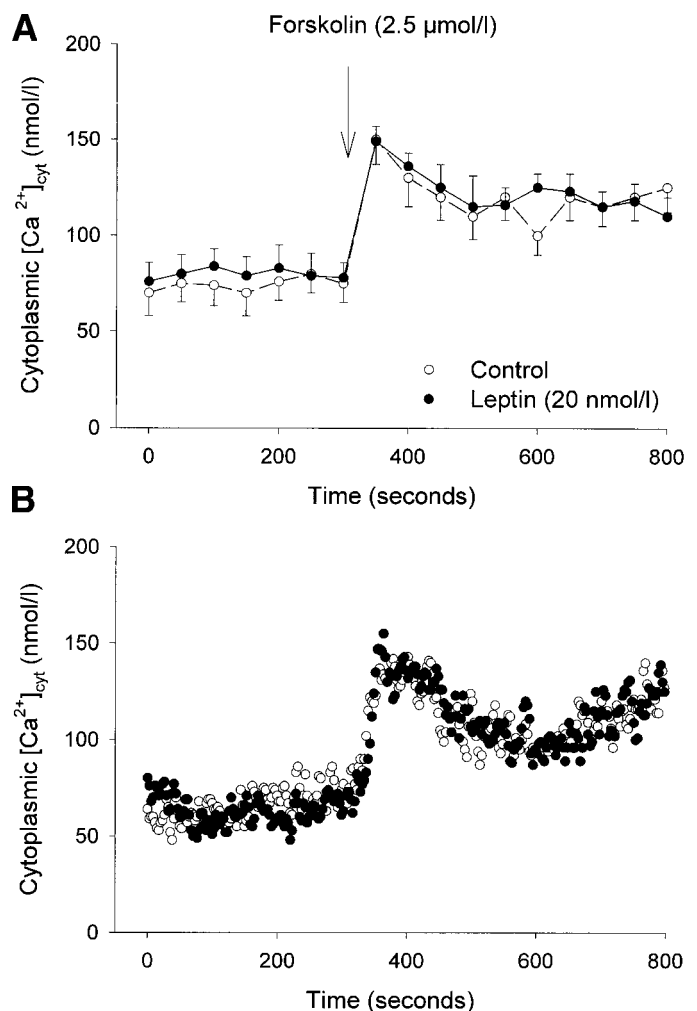

Fig. 6. Cytoplasmic concentration of calcium ($[Ca^{2+}]_{cyt}$) in fura 2-AM-loaded INS-1 cell suspensions at 10 mmol/l glucose with or without presence of leptin (20 nmol/l; present during entire experiment including 2-h loading period preceding measurement). At 300 s, forskolin (2.5 μmol/l) was added. In *A* means ± SE are shown ($n = 4$ for each series), and in *B* typical traces are shown.

glucose 5.5 mmol/l; 32), and in RINm5F cells (leptin 1 nmol/l; glyceraldehyde 15 mmol/l; 32). In contrast, leptin (0.7 and 7 nmol/l) stimulated insulin secretion in another study in HIT-T15 cells (glucose 7 mmol/l; 49). This variable and usually weak influence of leptin on insulin secretion suggests that leptin mainly influences signaling pathways other than those activated by glucose (or glyceraldehyde) in these cell lines. This is consistent with results in normal CD1 mouse islets, in which leptin had no effect at 16.7 mmol/l glucose but clearly inhibited insulin secretion if IBMX was added to the media (44). This is also similar to one study in islets isolated from wild-type littermates of *ob/ob* mice, in which leptin (20 and 100 nmol/l) did not inhibit glucose (20 mmol/l)-induced insulin secretion (9). However, another study has shown that leptin (10 nmol/l) inhibits glucose (16.7 mmol/l)-stimulated insulin secretion by 29% in wild-type *ob/ob* littermates (12).

Conflicting results have also been obtained in studies of glucose-stimulated insulin secretion in normal rat islets. Ishida and collaborators (24) demonstrated inhibition by leptin (8 nmol/l) at 5.5 but not at 11.1 mmol/l glucose (24); Kulkarni et al. (32) found inhibition by leptin (0.1 nmol/l) at 8 mmol/l glucose; and Seufert et al. (48) demonstrated inhibition by leptin (6.25 nmol/l) at 5.6 mmol/l glucose, whereas Ookuma et al. (42) showed inhibition by leptin (2.5 nmol/l) at high glucose (16.7 mmol/l). In contrast, Poitout and collaborators (44) did not find any influence of leptin (0.7 nmol/l) on glucose (16.7 mmol/l)-stimulated insulin secretion from normal rat islets and Ceddia and collaborators (8) demonstrated a stimulatory action of leptin (50 nmol/l) on insulin secretion in the presence of 2.8 or 5.6 mmol/l glucose in rat islets. Together, all of the studies examining the effects of leptin on glucose-stimulated secretion suggest that in some preparations leptin can inhibit glucose-stimulated insulin secretion, whereas in many others, leptin receptor activation does not appear to target the signaling pathways regulating glucose-stimulated insulin secretion. In a few other studies, leptin has even been found to stimulate insulin secretion.

Compared with the lack of a pronounced and consistent effect of leptin on glucose-stimulated insulin secretion from normal islets or insulin-producing cells, a marked inhibitory influence has been observed in islets isolated from *ob/ob* mice (9, 12, 29, 32), which do not produce biologically active leptin due to a mutation in the leptin gene (55). These results suggest that B cells from *ob/ob* mice may be more sensitive to the insulinostatic effects of exogenous leptin. This idea is supported by reports that the altered insulin secretion and islet function due to leptin deficiency are normalized by exogenous leptin (47).

Although most studies report an inhibitory action of leptin on insulin secretion, no general agreement exists regarding the mechanism of such an action. For example, one study in islets from *ob/ob* mice suggested that leptin acts primarily by opening the ATP-regulated K^+ channels, which would close voltage-sensitive Ca^{2+} channels and reduce the $[Ca^{2+}]_{cyt}$, thereby inhibiting the process of exocytosis (29). In contrast, another study of insulin-producing INS-1 cells revealed that, although leptin reduced $[Ca^{2+}]_{cyt}$, it did not affect membrane potential, suggesting that leptin works independently from K^+ channel activity (15). Other studies have shown that in isolated rat islets, leptin preferentially inhibits insulin secretion stimulated by PKC, activated either by acetylcholine (9) or a phorbol ester (42), suggesting a distally located action of leptin related to PKC-induced exocytosis. Yet other studies have demonstrated that leptin inhibits insulin secretion from rat islets and hamster insulinoma HIT-T15 cells induced by GLP-1 (14, 56). GLP-1 is thought to act primarily through receptors coupled to adenylate cyclase and increased intracellular cAMP (1, 10, 17). In another study, however, leptin did not inhibit GLP-1-induced insulin secretion in mouse islets (9). Collectively, these conflicting results suggest that the mechanism of the inhibitory action of leptin on insulin secretion is likely to differ in different types of insulin-producing cells. These differences may be ascribed to inherent characteristics of the cell types and/or to methodological differences between laboratories.

In contrast to the absence of an inhibitory action of leptin on glucose-stimulated insulin secretion, we found that leptin consistently and significantly inhibited insulin secretion stimulated by GLP-1, PACAP, forskolin, dibutyryl-cAMP, and IBMX. The similarity between the insulinotropic actions of these agents is that they all induce an increase in the intracellular content of cAMP: GLP-1, PACAP, and forskolin by stimulating adenylate cyclase and inducing formation of cAMP (1, 10, 17, 30, 50), dibutyryl-cAMP by being a membrane-penetrable analog of cAMP, and IBMX by reducing the degradation of cAMP through stimulation of PDE (25, 39). Therefore, in INS-1 cells, leptin appears to inhibit insulin secretion when the intracellular content of cAMP is elevated. This is similar to a recent study in isolated rat islets, in which leptin did not inhibit glucose-stimulated insulin secretion unless IBMX was included in the medium (44). Two possible mechanisms might explain this action of leptin. One explanation is that leptin reduces the cellular content of cAMP, for example by activating PDE. A second explanation is that leptin inhibits the downstream pathway initiated by activation of PKA. The former explanation would be supported by a recent study showing that leptin activates a specific subspecies of PDE, PDE3B, reducing cellular cAMP content in cultured neonatal rat islet monolayers and in HIT-T15 cells (56). However, we believe that the latter explanation is more likely because we found that leptin did not reduce the content of cAMP after stimulation with forskolin. It has also previously been shown that leptin does not affect the cellular content of cAMP in INS-1 cells, although leptin inhibits GLP-1-induced insulin secretion (14). Furthermore, leptin also inhibited the insulin response to IBMX, which nonspecifically inhibits all PDE subspecies, thereby precluding any specific action by leptin on these enzymes. The downstream action of activation of PKA involves phosphorylation of channels inhibiting

ATP-sensitive K^+ channels and/or activating Ca^{2+} channels, allowing a net uptake of calcium in addition to phosphorylation of exocytotic proteins (23, 25). We found that the peptide did not affect $[Ca^{2+}]_{cyt}$, either under baseline conditions or after increasing $[Ca^{2+}]_{cyt}$ after stimulation with forskolin. These results therefore suggest that leptin inhibits insulin secretion at a distal site after activation of PKA and that this site is also distal to changes in $[Ca^{2+}]_{cyt}$.

Our finding that leptin did not reduce $[Ca^{2+}]_{cyt}$ in the presence of 10 mmol/l glucose is at variance with the previous study by Fehmann and collaborators (15) who reported that leptin (10 nmol/l) reduced $[Ca^{2+}]_{cyt}$ in INS-1 cell suspensions (15). This discrepancy is probably explained by the difference in methodology used, because in their study the cells were incubated in a culture medium containing serum, whereas our study was performed keeping the cells in a well-defined serum-free incubation medium. This resulted in marked difference in baseline levels of $[Ca^{2+}]_{cyt}$ ($\approx$190 vs. $\approx$75 nmol/l in our present study). Hence, it is possible that leptin could reduce levels of $[Ca^{2+}]_{cyt}$ that have been elevated by factors included in the serum-containing medium. Our study shows, however, that under conditions of raised glucose and introduction of forskolin, when leptin inhibits insulin secretion, the $[Ca^{2+}]_{cyt}$ is unaffected by leptin.

In addition, we found that leptin did not affect the insulinotropic action of carbachol and TPA. Both these agents stimulate insulin secretion through activation of PKC (25). Thus these results suggest that, in INS-1 cells, leptin does not interact with this signaling pathway. This confirms a recent study in normal mouse islets, showing that leptin does not affect insulin secretion stimulated by acetylcholine or a phorbol ester (9). In this study it was also observed that in *ob/ob* mice islets, which exhibit increased sensitivity to agents activating PKC, leptin also suppresses the insulinotropic response to acetylcholine or the phorbol ester (9). This suggests that leptin has the capacity to inhibit PKC-induced insulin secretion, but this is only observed under abnormal conditions, such as in islets from *ob/ob* mice that have experienced chronic leptin deficiency and have increased sensitivity to PKC.

In our study, we also intended to use CCK-8, which has been demonstrated to stimulate insulin secretion in isolated rat islets through activation of PKC (26). However, CCK-8 did not stimulate insulin secretion in this cell line, which is similar to our recent experience with the RINm5F cell line (27). Hence, although CCK receptors are expressed (27), signaling pathways activated by CCK receptors seem not to be targeted in insulinoma cells.

On the basis of the results presented in this study, we conclude that leptin inhibits insulin secretion in INS-1 cells when the intracellular content of cAMP is elevated. A tentative model for mechanism of action of leptin is that leptin reduces the action of the cAMP-PKA signal transduction pathway, which may impair the function of proteins located further downstream in the signal transduction pathway that are phosphory-lated by PKA (25). This mechanism of leptin is different from that reported in other insulin producing cells (see above). It is likely that the inherent characteristics of insulin-producing cells, being of different origins and having different signaling characteristics, may help to explain discrepancies in the actions of leptin on insulin secretion. In addition, the extrinsic influence of the incubation conditions is another variable that needs to be considered.

Perspectives

Previous studies on the action and mechanism of leptin on insulin secretion have yielded mixed results, and this area of investigation remains controversial. Nonetheless, the main body of evidence suggests that leptin inhibits insulin secretion. We used the clonal cells, i.e., INS-1 cells, to examine whether the effect of leptin is dependent on primary mechanisms driving insulin secretion. We found that leptin inhibits insulin secretion under conditions when the cellular content of cAMP is elevated, but not when insulin secretion is stimulated by glucose alone or by agents that activate PKC. Because leptin did not reduce the cAMP content after stimulation with forskolin, it is suggested that in this cell line leptin inhibits insulin secretion by inhibiting the cAMP-PKA signaling pathway and not by activating the PDEs. However, more work is required to dissect the mechanisms in detail, and to determine the physiological relevance of leptin interactions with insulin secretion.

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