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Low, but Physiological, Concentration of GLP-1 Stimulates Insulin Secretion Independent of the cAMP-Dependent Protein Kinase Pathway

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Abstract. Glucagon-like peptide-1 (GLP-1) induces pancreatic insulin secretion via the cAMP-dependent protein kinase (PKA) pathway. However, the GLP-1 concentration used in the previous in vitro experiments was far from the in vivo concentrations. Alteration of plasma GLP-1 concentration at pM order lowers blood glucose concentration. In this study, we examined the GLP-1 action mechanism at a physiological concentration on insulin secretion. A high concentration of GLP-1 (10 nM) stimulated intracellular cAMP accumulation and insulin secretion was significantly inhibited by KT5720, a selective inhibitor of PKA. Low GLP-1 concentrations (1 pM) also increased insulin secretion without significant accumulation of intracellular cAMP, and KT5720 did not affect insulin secretion. Insulin secretion stimulated by 1 pM GLP-1 was reduced by inhibitors of calcium action, including verapamil, dantrolene, and BAPTA. Thus, we concluded that relatively low GLP-1 concentrations–comparable to in vivo blood concentrations–promoted insulin secretion independent of the cAMP-PKA pathway. This effect was dependent on intracellular Ca^{2+} concentration. The results of the present study may further the understanding of the dose-dependent response of GLP-1 signal transducing pathways and the complicated mechanism of insulin secretion. Studies of GLP-1 at physiologic concentrations may lead to new developments in studies of pancreatic β-cell function.

Keywords: glucagon-like peptide-1 (GLP-1), cAMP-dependent protein kinase pathway, insulin secretion, intracellular calcium, endoplasmic reticulum

Introduction

Glucagon-like peptide-1 (GLP-1) is the most potent promoter of insulin secretion in the body. As a result, GLP-1 analogs and dipeptidyl peptidase-IV (DPP-IV) inhibitors have been developed as drugs for the treatment of diabetes mellitus (1).

The glucose-lowering effect of GLP-1 is accompanied by increased insulin secretion in response to lowered blood glucose concentration, increased β-cell glucose sensitivity, decreased glucagon secretion, reduced insulin resistance, and delayed gastric emptying (2). The main mechanism of GLP-1-induced insulin secretion is generally thought to involve the cAMP-dependent protein kinase (PKA) pathway. Specifically, intracellular cAMP accumulates as a result of adenylate cyclase activation and subsequently activates PKA (3). Many studies have reported that GLP-1 increases intracellular cAMP levels. Various studies have identified calcium-induced calcium release (CICR) and ryanodine receptors as being involved after PKA activation (4). However, because the blood concentrations of substances examined in vivo differ from concentrations used in the in vitro experiments, the generalizability of the results of in vitro experiments to physiologic conditions remains controversial. Hormone actions often are concentration-dependent. For example, different concentrations of ghrelin elicit opposing actions: inhibition of insulin secretion at 10 nM and stimulation of insulin secretion at 1 pM (5, 6).

The concentration of GLP-1 in human peripheral blood is up to 30 pM, and even in the portal vein its concentration is 50 – 60 pM (7, 8). Thus, pancreatic cells are never exposed to GLP-1 concentrations in excess of

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60 pM. The highest concentration of GLP-1 analogue in blood after therapeutic subcutaneous administration is approximately 100 pM (1). GLP-1 does not reach nM concentrations in blood, even during treatment. The maximal fasting blood GLP-1 concentration is a few pM in mice and humans and the postprandial concentration does not exceed 10 pM. Even in rats, which have relatively high blood concentrations, the GLP-1 concentration does not exceed 100 pM. The postprandial GLP-1 concentration is only 20 pM, even in the presence of DPP-IV inhibitors (9). This slight change in GLP-1 concentration, however, has a large impact on blood glucose concentration.

The purpose of the present study was to clarify the mechanism of insulin secretion induced by physiological concentrations of GLP-1. We investigated the mechanism of GLP-1 action on insulin secretion at low concentrations (pM order) and compared it with the mechanism of insulin secretion induced by the high GLP-1 concentrations (nM order) that are typically used to study GLP-1 action. We previously demonstrated that the insulin secretory response of MIN6 cells to glucose and other secretagogues is similar to that of isolated islets (10). Therefore, MIN6 cells were used throughout this study instead of islet cells. The results of the present study clearly demonstrated that low GLP-1 concentrations promote insulin secretion independent of the cAMP-PKA pathway.

Materials and Methods

Reagents

Mouse insulin ELISA kits were purchased from Shibayagi (Gunma). cAMP EIA kits were purchased from GE Healthcare (Waukesha, WI, USA). Lactate dehydrogenase (LDH) cytotoxicity detection kits were purchased from Takara (Otsu). GLP-1 (7-36 Amide) was purchased from Peptide Institute, Inc. (Osaka). Dantrolene was purchased from Tocris Cookson Ltd. (Northpoint, UK). Verapamil was obtained from Nacalai Tesque, Inc. (Kyoto). All other chemicals were of analytical grade and were locally available.

MIN6 cell culture and insulin secretion analysis

MIN6 cells were kindly donated by Dr. Junichi Miyazaki at Osaka University (11). The cells (passages 20 – 30) were cultured in modified DMEM (containing 15% FBS, 5 μl/l 2-mercaptoethanol, 50 U/ml penicillin, 50 μg/ml streptomycin, 3.3 g/l $NaHCO_3$, and 25 mM glucose) in tissue culture flasks at 37°C under a humidified 5% CO_2 / 95% air atmosphere. The culture medium was removed and replaced with fresh medium every 24 h. Cells grown to 80% confluence were used in the experiments.

For determination of insulin secretion, MIN6 cells were cultured in 35-mm dishes containing 2 ml of culture medium for 72 h and then were preincubated for 30 min in 1 ml of Krebs-Ringer bicarbonate HEPES buffer (KRBH; 135 mM NaCl, 3.6 mM KCl, 2 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM HEPES, and 7.0 mM glucose; pH 7.4, 95% O_2 / 5% CO_2 saturated). The cells were pretreated with verapamil, dantrolene, and 1,2-bis(2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetraacetoxymethyl ester (BAPTA)-AM for 60 s before GLP-1 stimulation. Cells were pretreated with KT5720 20 min before GLP-1 stimulation. Following pretreatment, the buffer was exchanged for medium containing 1 pM GLP-1 KRBH with or without agents. The buffer was quickly collected by aspiration every 30 s with concurrent addition of fresh buffer. After buffer collection, the insulin remaining in the sample tube was confirmed to be less than the detection limit of the insulin ELISA. Ca^{2+}-free KRBH (135 mM NaCl, 3.6 mM KCl, 2 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1.0 mM EGTA, 10 mM HEPES, and 7.0 mM glucose; pH 7.4, 95% O_2 / 5% CO_2 saturated) was used to assess the influence of extracellular calcium. In this experiment, Ca^{2+}-free KRBH was added 60 s before GLP-1 stimulation. Insulin was measured using a mouse insulin ELISA kit.

Measurement of intracellular cAMP level

For determination of intracellular cAMP accumulation, MIN6 cells were treated in the same way as in the insulin secretion analysis. The reactions were stopped by ice-cold 6% trichloroacetic acid (TCA), and the cells were scraped off, sonicated, and centrifuged at 10,000 × *g* for 5 min at 4°C. Then obtained supernatants were washed 5 times with the water saturated diethyl-ether to remove TCA in the assay systems and cAMP concentrations were quantified using the cAMP EIA system (GE Healthcare UK, Ltd., Buckinghamshire, UK).

Protein assay

The content of protein in the alkaline-digested MIN6 cells was measured by the method of Lowry (12) using bovine serum albumin as standard.

Cytotoxicity study

Cell damage was quantified by measuring lactate dehydrogenase (LDH). LDH in the perfusate was measured using the LDH cytotoxicity detection kit in all experiments.

Statistical analyses

Results are presented as means ± S.D. The experimental numbers are given in the Fig. legends and each experiment was performed four times. The statistical analysis was determined by the method described in each Fig. legend following one-way analysis of variance.

Results

Effects of GLP-1 on insulin secretion and cAMP accumulation

Insulin secretion from MIN6 cells was significantly increased by 1 pM GLP-1. Insulin secretion was not dependent on the GLP-1 concentration over the range of 1 – 100 pM. However, at GLP-1 concentrations greater than 100 pM, insulin release was dose-dependent (Fig. 1). To demonstrate the appropriateness of our method of quantifying the cAMP level in MIN6 cells, forskolin-induced changes in intracellular cAMP levels were investigated. The intracellular cAMP level increased significantly after treatment with 100 nM forskolin to approximately 6-fold the control level (35 *vs* 6 pmol/mg protein, $P<0.0001$). Low concentrations of GLP-1 (up to 10 pM) failed to significantly increase the intracellular cAMP concentration. In contrast, intracellular cAMP was significantly increased by addition of 100 pM or more GLP-1 and the increase was GLP-1 dose-dependent (Fig. 1).

Thus, insulin secretion induced by less than 100 pM GLP-1 was not associated with intracellular cAMP accumulation, suggesting that the mechanism of GLP-1-induced insulin secretion is independent of the cAMP-PKA pathway.

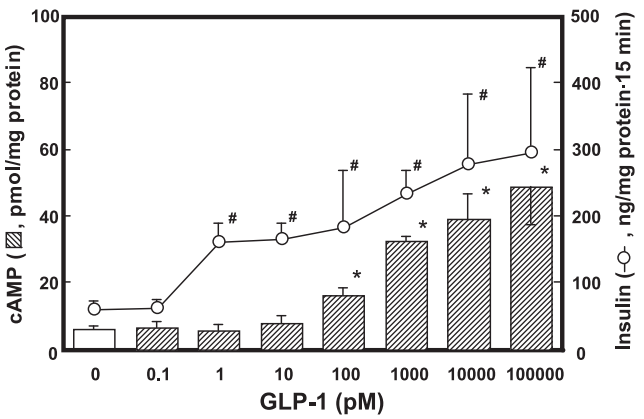

Fig. 1. Effects of GLP-1 on intracellular cAMP accumulation (bar) and insulin secretion (circle). Data are means ± S.D. of four independent experiments. **P*<0.05 and #*P*<0.05 *vs* each control (Dunnett's test). Control: GLP-1(–).

Effects of a PKA inhibitor on GLP-1–stimulated insulin secretion

Forskolin at 100 nM stimulated insulin secretion from MIN6 cells, and concomitant addition of PKA inhibitor, KT5720 (10 – 1000 nM), significantly inhibited insulin secretion induced by forskolin (Fig. 2A). Another PKA inhibitor, H89, also suppressed the forskolin effect on insulin secretion. As mentioned above, GLP-1 stimulated insulin secretion in a dose dependent manner at 100 pM – 100 nM. Addition of 100 nM KT5720 significantly, but not completely, inhibited GLP-1 stimulated

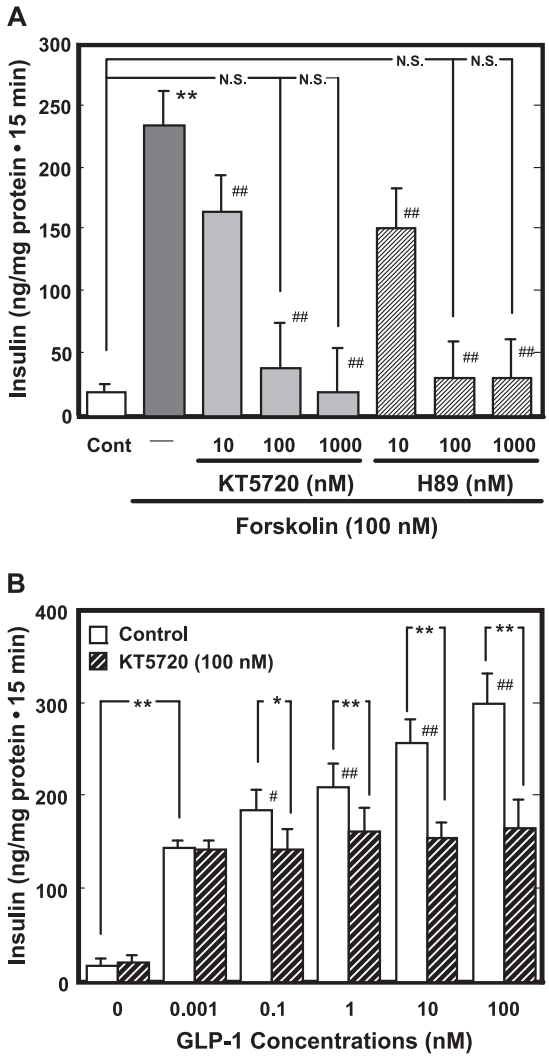

Fig. 2. Effects of protein kinase A inhibitors on forskolin and GLP-1 stimulated insulin secretion from MIN6 cells. A: Effects of protein kinase A inhibitors, KT5720 and H89, on insulin secretion from MIN6 cells stimulated by addition of 100 nM forskolin. Data are means ± S.D. of four independent experiments. ***P*<0.01 *vs* control. ##*P*<0.01 *vs* 100 nM forskolin. (Bonferroni's test). B: Effect of 100 nM KT5720 on GLP-1 stimulated insulin secretion from MIN6 cells. Data are means ± S.D. of four independent experiments. **P*<0.05 and ***P*<0.01 (Bonferroni's test). #*P*<0.01 and ##*P*<0.05 *vs* 0.001 nM GLP-1 (Bonferroni's test).

insulin secretion. In contrast, insulin secretion induced by 1 pM GLP-1 was not affected by addition of 100 nM KT5720 (Fig. 2B).

Effects of extra- and intra-cellular Ca^{2+} on GLP-1–stimulated insulin secretion

After stimulation with 1 pM GLP-1, insulin secretion increased markedly at 420 – 660 s (Phase 1), followed by oscillations in insulin release (Phase 2). GLP-1–stimulated insulin secretion was not inhibited by KT5720, but was completely inhibited by BAPTA, an intracellular Ca^{2+}-chelating agent (Fig. 3A).

The first half of Phase 1 insulin secretion was inhibited by addition of 3 μM verapamil. A similar waveform of insulin secretion was noted even in the absence of extracellular Ca^{2+} (Fig. 3B). The latter half of Phase 1 was inhibited by 1 μM dantrolene, and Phase 2 insulin secretion also was partially inhibited. Simultaneous addition of dantrolene and verapamil completely inhibited Phase 1 insulin secretion (Fig. 3C).

Discussion

The physiological actions of GLP-1 are mediated via the cAMP-PKA pathway, and GLP-1-induced insulin secretion is thought to be mediated by this pathway (2, 13, 14). However, involvement of other signal transducing pathways, including the cAMP-Epac-RyR cascade and PKC, has been reported (13, 15, 16). Nevertheless, elevated cAMP levels are thought to be required for all of these pathways. It should be noted that all of these mechanistic experiments were conducted using high GLP-1 concentrations (up to 1 nM). We observed that GLP-1 at relatively low concentrations–comparable to in vivo blood levels–promoted insulin secretion independent of the cAMP-PKA pathway. This effect was dependent on intracellular Ca^{2+} levels. These findings strongly suggest that the mechanism of GLP-1 action at low concentrations differs from that observed for high concentrations used in previous in vitro experiments.

Results from rat pancreas perfusion experiments revealed that GLP-1 stimulated insulin secretion at both 50 pM and 5 nM, thus demonstrating that GLP-1 induces insulin secretion at pM-order concentrations (8). Addition of a DPP-IV inhibitor, which potentiates insulin secretion by maintaining the blood GLP-1 concentrations (17), elevated the concentration of GLP-1 in blood by a few pM. These results strongly suggest that insulin secretion is sufficiently stimulated at relatively low concentrations (less than 100 pM) of GLP-1 in vivo.

Observation of intracellular cAMP dynamics with available techniques is difficult. The second messenger, cAMP, is constantly produced and degraded; and although the cAMP level may be altered, it rapidly

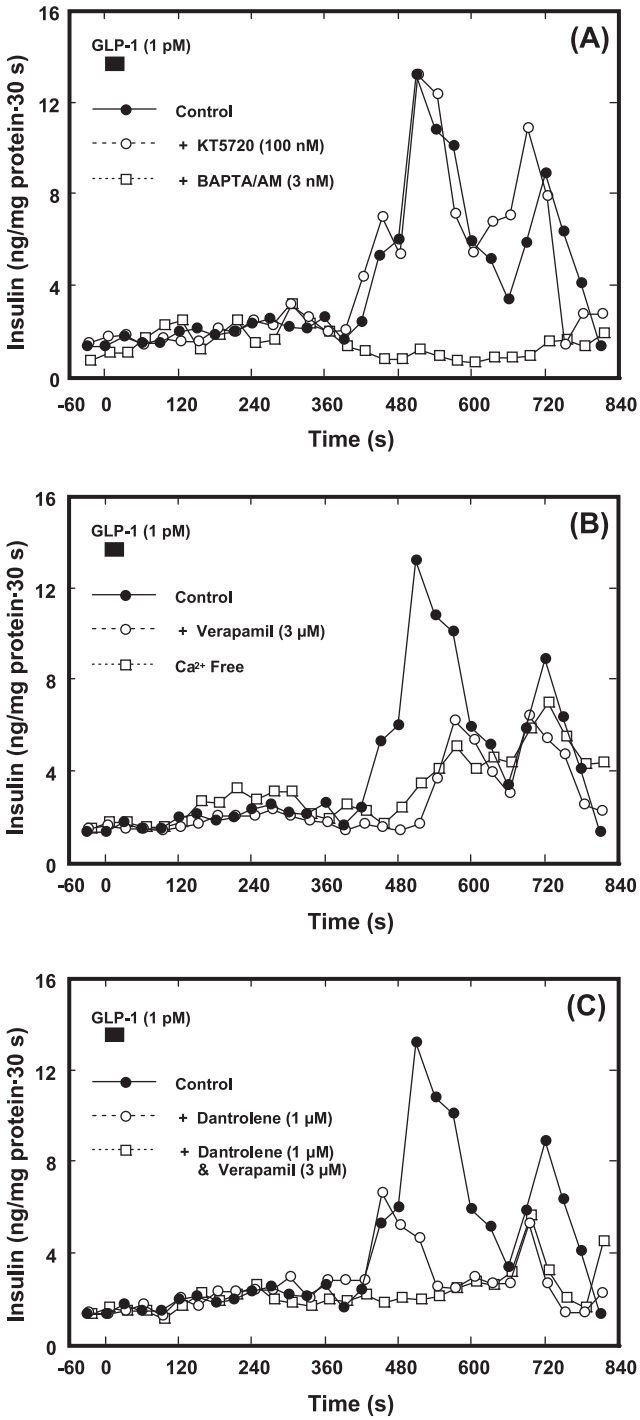

Fig. 3. Time course of insulin secretion from MIN6 cells stimulated by 1 pM GLP-1. A: Effects of 100 nM KT5720 and 3 nM BAPTA on 1 pM GLP-1–stimulated insulin secretion. B: Effects of 3 μM verapamil and extracellular Ca^{2+}-depletion on 1 pM GLP-1–stimulated insulin secretion. C: Effects of 1 μM dantrolene and 3 μM verapamil on 1 pM GLP-1–stimulated insulin secretion. Data are means ± S.D. of four independent experiments.

returns to the steady state value. Accordingly, experiments are generally performed in the presence of the phosphodiesterase (PDE) inhibitor isobuthylmethylxanthine (IBMX) to suppress cAMP degradation (4, 18). Although we first performed the experiments in the presence of IBMX, we recognized that IBMX itself affected the measurement system. As a result, the experiments described in this paper were performed without IBMX. A previous study noted that cAMP levels in the absence of IBMX did not necessarily reflect GLP-1 action because cAMP is constantly degraded by PDE (19). However, in the present study intracellular cAMP accumulation was detected without a PDE inhibitor. Theoretically, the observed cAMP level reflects the difference between production and degradation rates. Therefore, it should be possible to detect an increment if cAMP turnover is enhanced by GLP-1.

A relatively high concentration of GLP-1 (100 pM or more) and forskolin treatment significantly elevated the intracellular cAMP level in the absence of IBMX, and insulin secretion was increased proportionally. These results are consistent with previous reports (2, 20, 21). In similar experiments using IBMX, low GLP-1 concentrations (100 fM – 10 pM) did not increase cAMP levels, whereas cAMP was markedly elevated with nM-order GLP-1 (4). Considering the previous results with those of the present study, it is clear that a low concentration of GLP-1 does not elevate intracellular cAMP, regardless of the presence or absence of IBMX.

The results of the present study demonstrate that a relatively low, but physiological, concentration of GLP-1 induced insulin secretion that was not affected by inhibition of PKA. This finding strongly suggests a cAMP-PKA–independent pathway. Complete inhibition of GLP-1 action by an intracellular Ca^{2+} chelating agent (BAPTA) indicates that intracellular Ca^{2+} elevation is required for GLP-1 stimulation of insulin secretion. Because a voltage-dependent Ca^{2+} channel inhibitor, verapamil, and a ryanodine receptor (RyR) inhibitor, dantrolene, inhibited insulin secretion, GLP-1–induced insulin secretion involves both extracellular Ca^{2+} influx and intracellular Ca^{2+} release from the endoplasmic reticulum (ER), as well as from CICR.

The most important finding of the present study is the possibility that the signal-transducing pathway of GLP-1 action on pancreatic insulin secretion involves both cAMP-PKA–dependent and –independent mechanisms, which are dependent on GLP-1 concentration. This mode of action is not specific to pancreatic β cells. Similar findings have been reported in adipocytes. A relatively low GLP-1 concentration (pM order) promoted lipogenesis without intracellular cAMP accumulation, while a relatively high GLP-1 concentration (nM order) stimulated lipolysis with cAMP accumulation (22 – 24).

In conclusion, the present study suggested the possibility that physiological concentrations of GLP-1 may act through a cAMP-PKA–independent pathway to stimulate insulin secretion from pancreatic β cells. The results of the present study may further our understanding of the dose-dependent effects of GLP-1 action and the complicated mechanism of insulin secretion. Studies using physiologic GLP-1 concentrations may lead to new developments in the study of pancreatic β cell function.

Acknowledgments

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