Glucagon-Like Peptide-1 Enhances Glucokinase Activity in Pancreatic β-Cells through the Association of Epac2 with Rim2 and Rab3A

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Glucokinase (GK), which phosphorylates D-glucose, is a major glucose sensor in β-cells for glucose-stimulated insulin secretion (GSIS) and is a promising new drug target for type 2 diabetes (T2D). In T2D, pancreatic β-cells exhibit defective glucose sensitivity, which leads to impaired GSIS. Although glucagon-like peptide-1-(7–36)-amide (GLP-1) is known to enhance β-cell glucose sensitivity, the effect of GLP-1 on GK activity is still unknown. The present study demonstrated that GLP-1 pretreatment for 30 min significantly enhanced GK activity in a glucose-dependent manner, with a lower Michaelis-Menten constant (K_m) but unchanged maximal velocity (V_{max}). Thus, GLP-1 acutely enhanced cellular glucose uptake, mitochondrial membrane potential, and cellular ATP levels in response to glucose in rat INS-1 and native β-cells. This effect of GLP-1 occurred via its G protein-coupled receptor pathway in a cAMP-dependent but protein kinase A-independent manner with evidence of exchange protein activated by cAMP (Epac) involvement. Silencing Epac2, interacting molecule of the small G protein Rab3 (Rim2), or Ras-associated protein Rab3A (Rab3A) significantly blocked the effect of GLP-1. These results suggested that GLP-1 can further potentiate GSIS by enhancing GK activity through the signaling of Epac2 to Rim2 and Rab3A, which is the similar pathway for GLP-1 to potentiate Ca^{2+}-dependent insulin granule exocytosis. The present finding may also be an important mechanism of GLP-1 for recovery of GSIS in T2D. **(*Endocrinology* 153: 574–582, 2012)**

In pancreatic β-cells, defective insulin secretion in response to glucose is a characteristic of overt type 2 diabetes (T2D) (1, 2). Impaired glucose sensitivity may further threaten β-cell survival due to a disturbed utilization of glucose as a cellular energy source (3). This impairment of diabetic β-cells may be due to disturbed glucose metabolism in mitochondria (4). In addition, some diabetic animals, such as db^+/db^+ mice and obese Zucker rats, exhibit an impairment of substantial β-cell glucose uptake (5), which may result from gradual reductions of glucokinase (GK, hexokinase type IV) and glucose transporter 2 protein levels (6). Loss-of-function and gain-of-function mutations of GK lead to several types of hereditary T2D, such as maturity-onset diabetes of the young and permanent neonatal diabetes mellitus (7) and hypoglycemia (8), respectively, suggesting that GK activity and GK protein levels are critical for normal glucose sensitivity of β-cells (9).

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Abbreviations: Epac, Exchange protein activated by cAMP; FBS, fetal bovine serum; GK, glucokinase; GLP-1, glucagon-like peptide-1-(7–36)-amide; GLP-1R, G protein-coupled GLP-1 receptor; GLUT2, glucose transporter type 2; GSIS, glucose-stimulated insulin secretion; G-6-P, glucose 6-phosphate; KRPH, Krebs-Ringer phosphate-HEPES; $\Delta\psi m$, mitochondrial membrane potential; NAD, nicotinamide adenine dinucleotide; 8-pCPT-2′-*O*-Me-cAMP-AM, 8-(4-chlorophenylthio)-2′-*O*-methyladenosine-3′,5′-cyclic monophosphate-acetoxymethyl ester; PKA, protein kinase A; Rab3A, Ras-associated protein Rab3A; Rim2, interacting molecule of the small G protein Rab3; siRNA, small interfering RNA; Sp-6-Bnz-cAMP, N^6-benzoyl-adenosine-3′,5′-cyclic monophosphorothioate, Sp-isomer; T2D, type 2 diabetes; TMRE, tetramethylrhodamine ethyl ester perchlorate; V_{max}, maximal velocity.

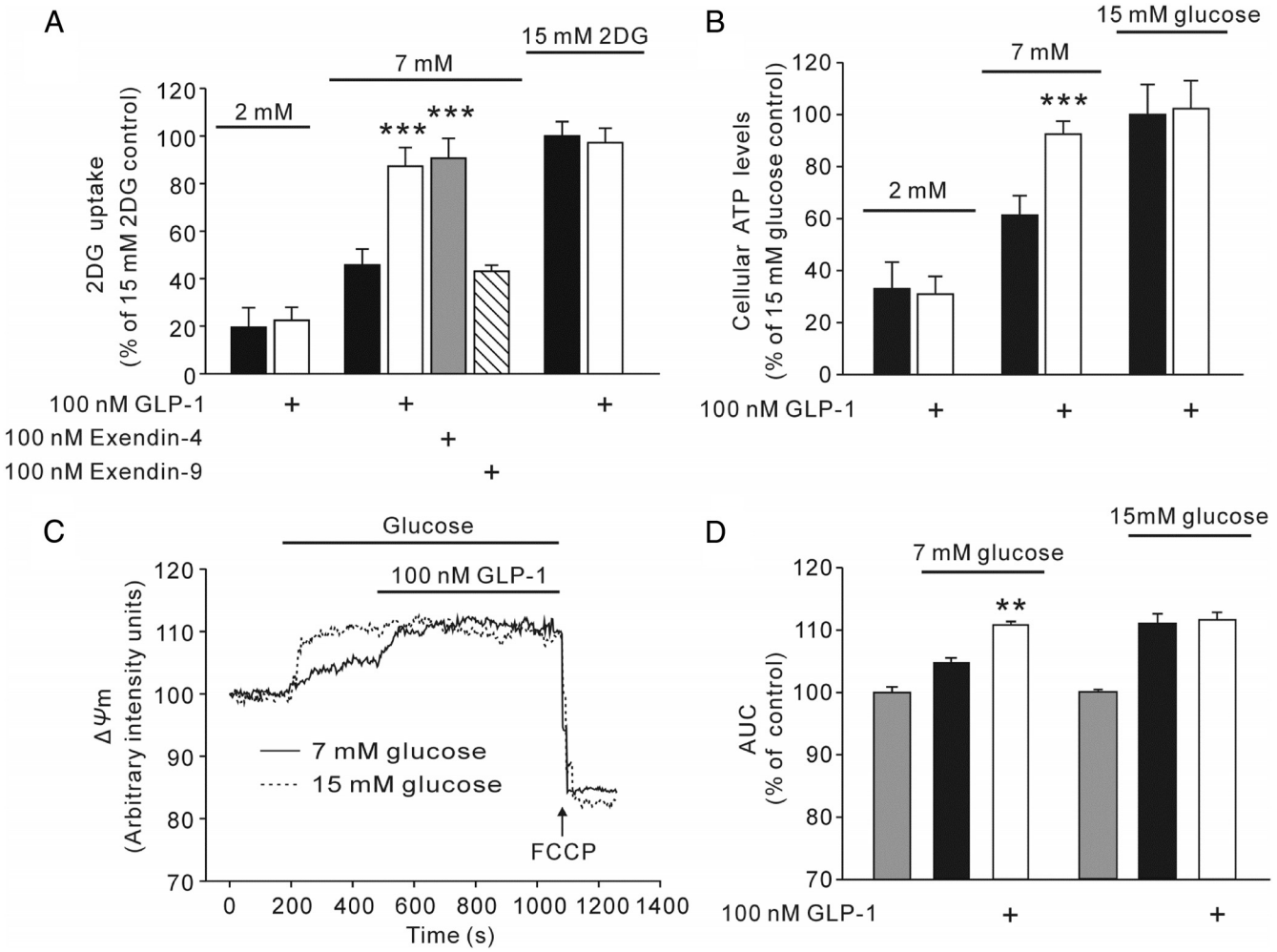

FIG. 1. Effect of GLP-1 on 2-deoxy-[^{3}H]glucose uptake, cellular ATP levels and $\Delta\psi$m in INS-1 cells. A, After incubation in KRPH buffer for 30 min, INS-1 cells were pretreated with 100 nM GLP-1, 100 nM exendin-4, or 100 nM exendin-9 for 20 min and then exposed to a mixture of 2-deoxy-[^{3}H]glucose (2DG) and 2, 7, or 15 mM unlabeled 2-deoxyglucose for 10 min. B, After incubation in KRPH buffer for 30 min, INS-1 cells were pretreated with or without 100 nM GLP-1 for 20 min and then exposed to 2, 7, or 15 mM glucose for 10 min. Cellular ATP concentration was assayed by luciferase measurement. C, After establishment of a stable $\Delta\psi$m baseline, INS-1 cells were stimulated with 7 or 15 mM glucose for 5 min, and then 100 nM GLP-1 was applied. After 10 min, the protonophore carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP, 2 μM) was added. Images were collected at 3-sec intervals, and results are plotted as the change in fluorescence intensity in arbitrary units. Representative data were chosen from six independent experiments. D, Area under the curve (AUC) of each area was depicted as the percentage of each control value. Values represent mean ± SE; n = 6–8 per group. **, $P < 0.01$; ***, $P < 0.001$, comparison with each control value.

Glucagon-like peptide-1-(7–36)-amide (GLP-1), an incretin hormone secreted by endocrine L cells of the intestinal tract, increases glucose-stimulated insulin secretion (GSIS) and cell survival in normal and diabetic β-cells (10). GLP-1 and its analogs have been implicated in the restoration of glucose-resistant β-cells to a state of glucose competence (11–13). In this process, activation of the G protein-coupled receptor of GLP-1 (GLP-1R) inhibits ATP-sensitive K^+ (K_{ATP}) (14) and voltage-dependent K^+ (15) channels and activates voltage-dependent Ca^{2+} channels (16) in a glucose-dependent manner. Several reports have suggested that GLP-1R signaling further mobilizes cytosolic Ca^{2+} from the endoplasmic reticulum, thereby stimulating mitochondrial glucose metabolism (17). Moreover, to potentiate Ca^{2+}-mediated insulin granule exocytosis, GLP-1 signaling directly targets insulin granule-associated proteins, such as Rim, an interacting molecule of the small G protein Rab3, and Piccolo, a Ca^{2+}-binding cytoskeletal matrix protein that associates with the active zone (18–20). Although prolonged treatment with the GLP-1R agonist exendin-4 has been shown to increase GK protein levels in β-cells (21), it is unknown whether GLP-1 affects GK activity to sensitize β-cells to glucose.

We have previously reported (22) that GLP-1 acutely restores 2-deoxyglucose uptake impaired by glucosamine, a glucose uptake inhibitor (23, 24), in rat INS-1 and native β-cells in a cAMP-dependent but protein kinase A (PKA)-independent manner. In the present study, we further showed that the effect of GLP-1 was due to its potentiation of GK activity through GLP-1R signaling employing

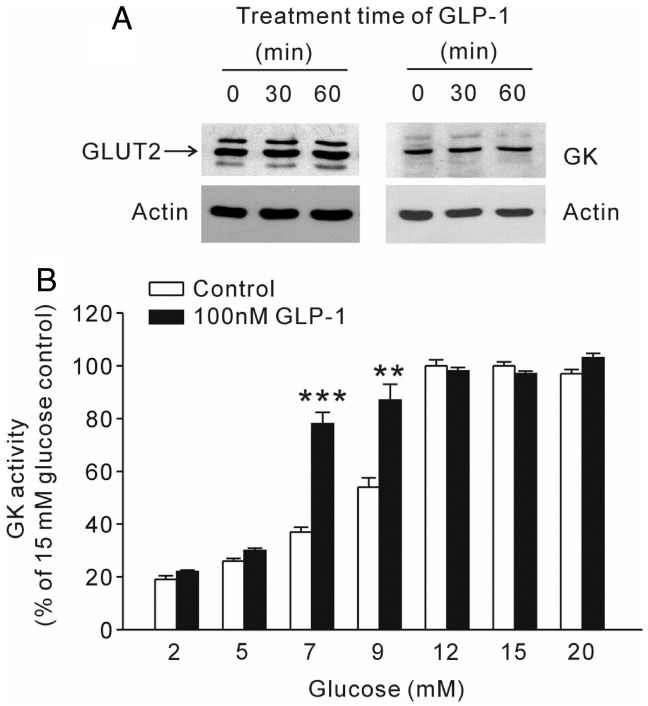

FIG. 2. Effect of GLP-1 on GLUT2 and GK in INS-1 cells. A, Cells were exposed to 100 nM GLP-1 for 30 or 60 min. GLUT2 and GK expression levels were examined with Western blot analysis (representative data chosen from four independent experiments). B, After treatment with or without 100 nM GLP-1 for 30 min, GK activity was determined at concentrations of 2, 5, 7, 9, 12, 15, and 20 mM glucose in INS-1 cell lysates. Values represent mean ± SE; n = 6 per group. **, $P < 0.01$; ***, $P < 0.001$, comparison with each control value.

cAMP-regulated guanine nucleotide exchange factors, which are also known as exchange proteins activated by cAMP (Epac) (19, 25). The present results also suggest that cAMP-dependent Epac2 activation involves interacting molecule of the small G protein Rab3 (Rim2) and Ras-associated protein Rab3A (Rab3A) when potentiating GK activity, which is a similar pathway to that of GLP-1 when facilitating Ca^{2+}-dependent insulin granule exocytosis.

Materials and Methods

Reagents

Fetal bovine serum (FBS) was purchased from Life Technologies, Inc. (Carlsbad, CA). RPMI 1640 medium was purchased from Welgene (Daegu, Korea). The nonspecific dipeptidyl peptidase IV inhibitor (10 μM) purchased from Linco Research (St. Charles, MO) was coadministered with GLP-1. LY294002 was purchased from Cell Signaling Technology (Beverly, MA). H-89 and AG1478 were purchased from Cayman Chemical (Ann Arbor, MI). MDL-12330A and PP1 were purchased from Biomol (Plymouth Meeting, PA). 8-(4-Chlorophenylthio)-2′-*O*-methyladenosine-3′,5′-cyclic monophosphate-acetoxymethyl ester (8-pCPT-2′-*O*-Me-cAMP-AM) and N^6-benzoyl-adenosine-3′,5′-cyclic monophosphorothioate, Sp-isomer (Sp-6-Bnz-cAMP) were purchased from Biolog (Bremen, Germany). 2-Deoxy-[^{3}H]glucose (10 Ci/mmol) was purchased from PerkinElmer Life and Analytical Science (Waltham, MA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Table 1. Kinetic parameters for GK in INS-1 cells

	V_{max} (mol/kg DNA·90 min)	K_m (mM glucose)
With GLP-1	7.86 ± 0.21	12.7 ± 0.21
Without GLP-1	7.98 ± 0.16	10.6 ± 0.49[a]

After treatment with or without 100 nM GLP-1 for 30 min, GK V_{max} and K_m were determined at concentrations of 2–100 mM glucose in INS-1 cell lysates. V_{max} and K_m values of GK were calculated as described in the text. Values represent mean ± SE; n = 6 per group.

[a] $P < 0.05$ represents a comparison with control value without GLP-1.

INS-1 cell culture and isolation of islets

Rat insulinoma INS-1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 50 U/ml penicillin, and 0.1 mg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37 C. Islets of Langerhans were isolated from the pancreas of male Sprague Dawley rats using a collagenase digestion technique. To collect dispersed islet cells, the islets were further triturated and incubated in RPMI 1640 medium with 11.1 mM glucose, 10% FBS in a humidified incubator at 37 C with 5% CO_2 for 24 h. The dispersed islet cells were stained with insulin and glucagon antibodies to confirm purity of β-cells. All procedures were submitted and approved by the Institutional Guideline Committee for Animal Experiments.

Measurement of 2-deoxy-[^{3}H]glucose uptake

INS-1 cells cultured in 35-mm tissue culture dishes at a density of 1×10^6 cells per well were washed with and incubated in Krebs-Ringer phosphate-HEPES (KRPH) buffer [10 mM phosphate buffer (pH 7.4), 1 mM $MgSO_4$, 1 mM $CaCl_2$, 136 mM NaCl, 4.7 mM KCl, and 10 mM HEPES (pH 7.6)] containing 0.2% BSA and 2 mM glucose for 30 min. After pretreatment with or without 100 nM GLP-1 for 20 min, glucose uptake was determined by adding a mixture of 2-deoxy-[^{3}H]glucose (1 μCi; final concentration, 0.1 μM) and 2, 7, or 15 mM unlabeled 2-deoxyglucose. After 10 min incubation, the reaction was stopped by three quick washes with ice-cold PBS. The total treatment time of GLP-1 was 30 min. The cells were then lysed in PBS containing 0.2 M NaOH, and glucose uptake was assessed by scintillation counting.

ATP measurement

Cellular ATP concentration was assayed by luciferase measurement (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. INS-1 or dispersed islet β-cells in 35-mm tissue culture dishes at a density of 1×10^6 cells per well were incubated for 30 min in KRPH buffer containing 0.2% BSA and 2 mM glucose. After pretreatment with or without 100 nM GLP-1 for 20 min, cells were exposed to 2, 7, or 15 mM glucose for 10 min at 37 C. The total treatment time of GLP-1 was 30 min. The cells were then harvested, and all procedures to measure ATP were performed at 28 C.

GK activity determination

GK activity was determined by following the accumulation of reduced nicotinamide adenine dinucleotide (NAD) at 340 nm

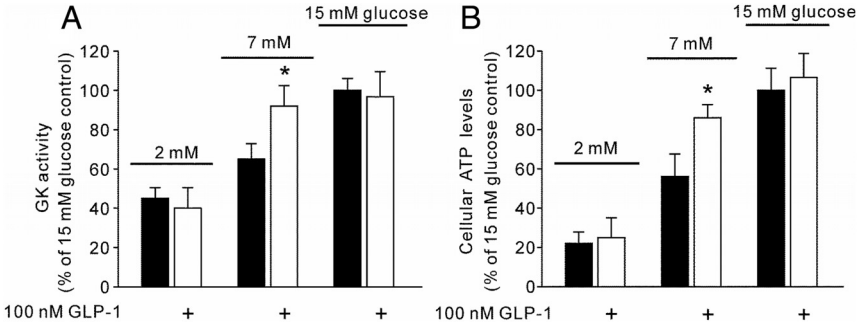

FIG. 3. Effect of GLP-1 on GK activity and cellular ATP levels in dispersed islet β-cells. A, After treatment with or without 100 nM GLP-1 for 30 min, GK activity was determined at concentrations of 2, 7, and 15 mM glucose in dispersed islet β-cell lysates. B, After incubation in KRPH buffer for 30 min, dispersed islet β-cells were pretreated with or without 100 nM GLP-1 for 20 min and then exposed to 2, 7, or 15 mM glucose for 10 min. Cellular ATP concentration was assayed by luciferase measurement. Values represent mean ± SE; n = 6–8 per group. *, $P < 0.05$, comparison with each control value.

using a spectrophotometer. In this assay, GK phosphorylates glucose to yield glucose 6-phosphate (G-6-P), which is transformed into 6-phosphogluconate, and NAD is then reduced by G-6-P dehydrogenase from *Leuconostoc mesenteriode* (Sigma). This last reaction depends directly on GK activity. Briefly, INS-1 or dispersed islet β-cells were incubated for 30 min in KRPH buffer containing 0.2% BSA and 2 mM glucose. After pretreatment with or without 100 nM GLP-1 for 30 min, cells were harvested and lysed in 500 μl lysis buffer [1 μM phenylmethylsulfonyl fluoride, 50 mM Tris (pH 7.6), 4 mM EDTA, 150 mM KCl, 4 mM Mg_2SO_4, and 2.5 mM dithiothreitol] and centrifuged at 4 C for 10 min at 12,000 × *g* to remove mitochondrial-bound hexokinase. DNA content was measured using 10-μl aliquots of the extract. The supernatant (10 μl) was added to 200 μl reaction buffer containing 50 mM HEPES/HCl (pH 7.6), 100 mM KCl, 7.4 mM $MgCl_2$, 15 mM β-mercaptoethanol, 0.5 mM NAD^+, 0.05% BSA (wt/vol), 3 μg/ml G-6-P dehydrogenase, and 5 mM ATP with glucose (2–100 mM). The assay was conducted for 90 min at 30 C, and the reaction was stopped by adding 1 ml of a 500 mM $NaHCO_3$ buffer (pH 9.4). Absorbance was measured in triplicate samples (excitation 350 nm/emission 460 nm), and the mean value was used as a single observation. For the standard curve, 0.3–3.0 nmol G-6-P was used in the reaction buffer containing 100 mM glucose. Maximal velocity (V_{max}) and Michaelis-Menten constant (K_m) of GK were obtained by Lineweaver-Burk plot.

Confocal microscopy to measure mitochondrial membrane potential (Δψm)

Mitochondria were labeled using the mitochondria-specific dye tetramethylrhodamine ethyl ester perchlorate (TMRE; Invitrogen). INS-1 or dispersed islet β-cells were incubated for 30 min in KRPH buffer containing 0.2% BSA, 2 mM glucose, and 10 nM TMRE. Confocal microscopy was performed on living cells using a Carl Zeiss confocal microscope (LSM 5 EXCITER; Carl Zeiss, Jena, Germany) connected to an axio-observer Z1 inverted microscope using a C-Apochromat ×40 objective (1.2 numerical aperture). Fluorescent images were generated at 37 C, and all solutions contained 10 nM TMRE during recordings. TMRE fluorescence was excited at 514 nm and recorded at 590 nm. For measurements of the time-dependent TMRE fluorescence changes, data were acquired every 3 sec. The relationship between TMRE fluorescence and Δψm is governed by the Nernst equation. The Δψm-dependent component of TMRE will accumulate in a Nernstian fashion that can be described by the intensity of its fluorescence (26). The non-Δψm-dependent component of TMRE, also known as the binding component, was ignored because it is fixed and voltage independent (26). Image analysis was completed with LSM 5 EXCITER software (Carl Zeiss).

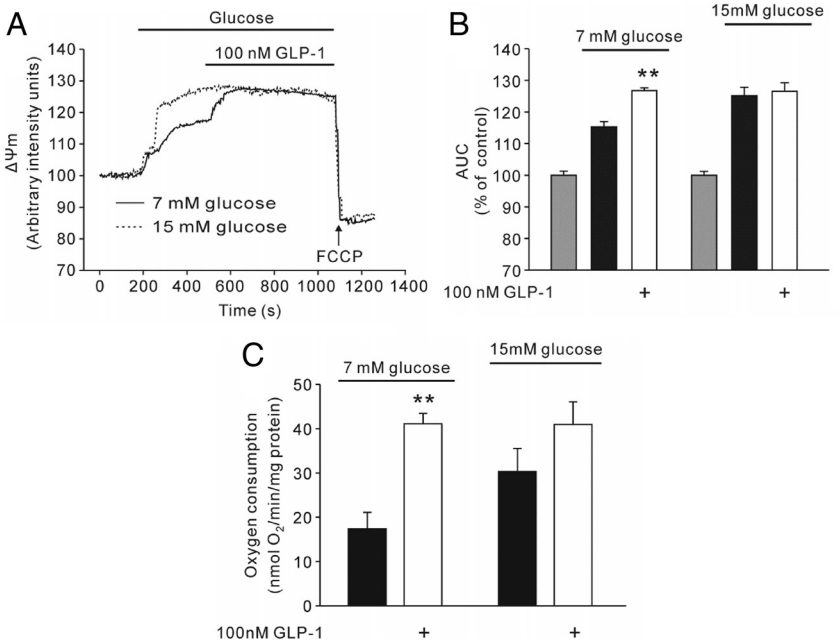

FIG. 4. Effect of GLP-1 on ΔΨm and respiration in dispersed islet β-cells. A, After establishment of a stable Δψm baseline, dispersed islet β-cells were stimulated with 7 or 15 mM glucose for 5 min, and then 100 nM GLP-1 was applied. After 10 min, *p*-(trifluoromethoxy)phenylhydrazone (FCCP; 2 μM) was added. Images were collected at 3-sec intervals, and results were plotted as the change in fluorescence intensity in arbitrary units. Representative data were chosen from six independent experiments. B, Area under the curve (AUC) of each area was depicted as the percentage of each control value. C, Oxygen consumption was measured with a Clark-type oxygen electrode. After treatment with 7 or 15 mM glucose for 7 min, dispersed islet cells were exposed to 100 nM GLP-1 for 10 min. Oxygen uptake is expressed in nanomoles of O_2 per minute per milligram of protein. Values represent mean ± SE; n = 6–8 per group. **, $P < 0.01$, comparison with each control value.

Oxygen consumption

Oxygen uptake by dispersed islet cells was measured with a Clark-type oxygen

electrode (Instech, Plymouth Meeting, PA) in a 600-μl air-saturated chamber at 37 C. Respiration medium consisted of 10 mM phosphate buffer, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 136 mM NaCl, 4.7 mM KCl, and 10 mM HEPES, 0.2% BSA (pH 7.4). Endogenous (basal) respiration was measured for 3 min. After treatment with 7 or 15 mM glucose for 7 min, dispersed islet cells were exposed to 100 nM GLP-1 for 10 min. Oxygen uptake is expressed in nanomoles of O_2 per minute per milligram of protein.

Western blot analysis

INS-1 cells were incubated for 30 min in KRPH buffer containing 0.2% BSA and 2 mM glucose. After pretreatment with or without 100 nM GLP-1 for 30 or 60 min, the cells were washed twice in ice-cold PBS, and total cellular proteins were extracted in lysis buffer [10 mM Tris-Cl (pH 7.4), 130 mM NaCl, 5% (vol/vol) Triton X-100, 5 mM EDTA, 200 nM aprotinin, 20 mM leupeptin, 50 mM phenanthroline, and 280 mM benzamidine-HCl] for 20 min at 4 C. The protein concentrations were measured using the Bio-Rad (Hercules, CA) protein assay. Cellular lysates were separated by SDS-PAGE and electrotransferred to an Immobilon-P membrane (Millipore, Billerica, MA). The membranes were then probed with specific antibodies as follows: anti-GK (Santa Cruz), anti-glucose transporter type 2 (GLUT2) (Santa Cruz) targeting amino acids in the extracellular loop, anti-Epac2 (Santa Cruz), anti-Rim2 (Abcam, Cambridge, UK), anti-Rab3A (Santa Cruz), and anti-β-actin (Sigma). The immunoreactive bands were visualized with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz) using enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK). The experiments were repeated at least three times.

Small interfering RNA (siRNA) transfection

Knockdown of Epac2, Rim2, and Rab3A expression in INS-1 cells was performed using BLOCK-iT RNAi duplexes (Invitrogen). The siRNA sequences for the target genes were as follows: GCTACTACAGGAGCCAGCCATTCAA (Epac2), AGACAA TGATCTGTAACACCTTCCC (Rim2), and GCGCCAAGGA CAACATTAATGTCAA (Rab3A). Cells were transfected with 50 nM siRNA using the Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. Stealth RNAi negative control (Invitrogen) was used as a control for off-target effects. The knockdown efficiency was assessed by Western blot analysis. The cells were treated as indicated in the figure legends and processed for 2-deoxyglucose uptake, cellular ATP level, or GK activity analyses.

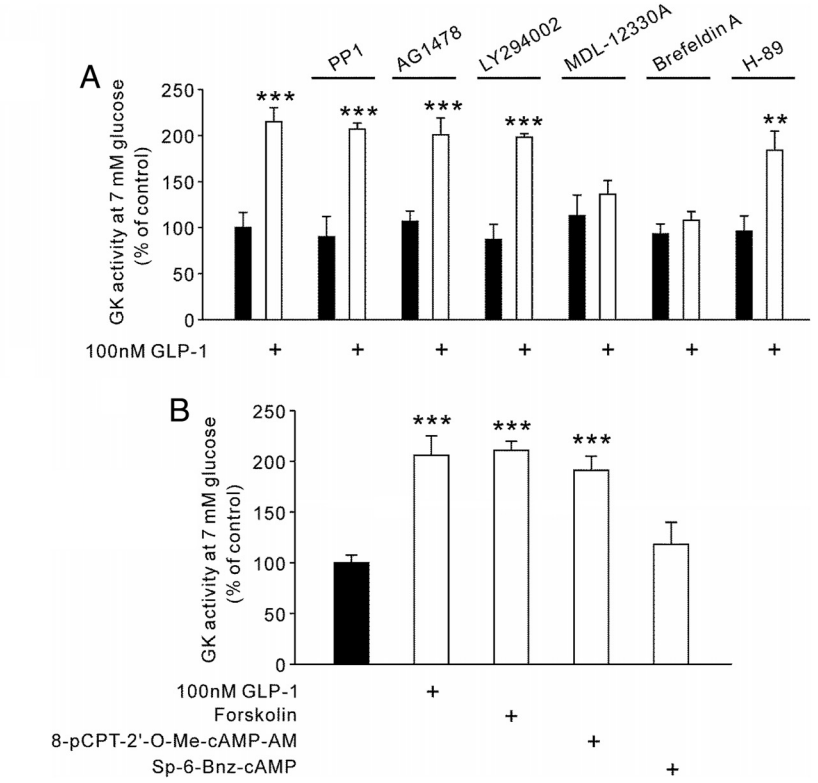

FIG. 5. Involvement of cAMP and Epac in the GLP-1 effects on GK activity in INS-1 cells. A, INS-1 cells were incubated in KRPH buffer for 30 min with the following inhibitors as indicated: c-Src inhibitor PP1 (10 μM), epidermal growth factor receptor kinase inhibitor AG1478 (250 nM), phosphoinositide 3-kinase inhibitor LY294002 (20 μM), adenylate cyclase inhibitor MDL-12330A (10 μM), nonselective Epac inhibitor brefeldin A (10 μg/ml), or nonselective PKA inhibitor H-89 (10 μM). The cells were further incubated for 30 min with or without 100 nM GLP-1. GK activity was determined at 7 mM glucose in INS-1 cell lysates. B, INS-1 cells were incubated in KRPH buffer for 30 min. The cells were treated with the following agents for 20 min: GLP-1, cAMP-elevating agent forskolin (100 μM), Epac-selective activator 8-pCPT-2′-*O*-Me-cAMP-AM (100 μM), or PKA-selective activator Sp-6-Bnz-cAMP (100 μM). GK activity was determined at 7 mM glucose in INS-1 cell lysates. Values represent mean ± SE; n = 6–8 per group. **, $P < 0.01$; ***, $P < 0.001$, comparison with each control value.

Statistical analysis

The results are expressed as mean ± SE. SPSS version 14.0 (SPSS Inc., Chicago, IL) was used for the statistical analyses. The area under the curve was calculated using Microcal (Northampton, MA) Origin software version 7.0. Comparisons between two groups were performed with the Student's two-tailed *t* test for paired or unpaired data. For comparisons of more than two groups, significance was tested using ANOVA with Bonferroni correction to deal with relatively small numbers of samples. *P* values <0.05 were considered significant.

Results

GLP-1-mediated increase in 2-deoxy-[^{3}H]glucose uptake

As shown in Fig. 1A, a 20-min pretreatment with 100 nM GLP-1 increased 2-deoxyglucose uptake in INS-1 cells when using 7 mM 2-deoxyglucose, and the effect was not detected when using 15 mM 2-deoxyglucose. The effect of GLP-1 at 7 mM 2-deoxyglucose was replicated

by the GLP-1R agonist exendin-4. However, pretreatment with the GLP-1R antagonist exendin-9 had no effect.

GLP-1-mediated increase in cellular ATP level and $\Delta\psi m$

Cellular ATP levels were measured in INS-1 cells at various concentrations of glucose with or without pretreatment with 100 nM GLP-1 (Fig. 1B). The ATP levels at 7 mM glucose were greater in the GLP-1-pretreated group. The effect of GLP-1 was not detected when using 15 mM glucose, which was consistent with the 2-deoxyglucose uptake results. Consistently, acute treatment with 100 nM GLP-1 further increased $\Delta\psi m$ in response to 7 mM glucose but not 15 mM glucose (Fig. 1, C and D).

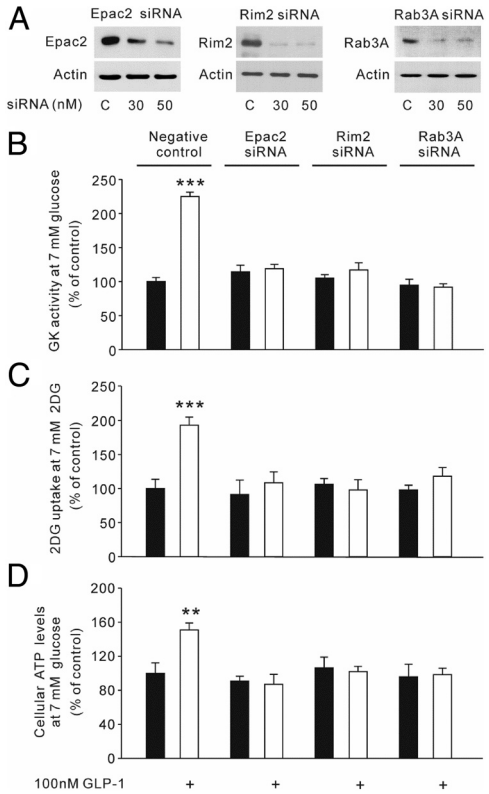

FIG. 6. Effect of GLP-1 on GK activity, 2-deoxy-[^{3}H]glucose uptake, and cellular ATP levels in Epac2-, Rim2-, or Rab3A-knockdown INS-1 cells. INS-1 cells were transfected with BLOCK-iT RNAi (Epac2, Rim2, or Rab3A siRNA) for 72 h. A, Western blots of transfected cells were quantified. B, Transfected cells were incubated in KRPH buffer for 30 min. After treatment with or without 100 nM GLP-1 for 30 min, GK activity was determined at 7 mM glucose in INS-1 cell lysates. C, Transfected cells were incubated in KRPH buffer for 30 min. After treatment with or without 100 nM GLP-1 for 20 min, the cells were exposed to a mixture of 2-deoxy-[^{3}H]glucose (2DG) and 7 mM unlabeled 2-deoxyglucose for 10 min. D, Transfected cells were incubated in KRPH buffer for 30 min. After treatment with or without 100 nM GLP-1 for 20 min, the cells were exposed to 7 mM glucose for 10 min. Cellular ATP concentration was assayed by luciferase measurement. Values represent mean ± SE; n = 6–8 per group. **, $P < 0.01$; ***, $P < 0.001$, comparison with each control value.

GLP-1-mediated increase in GK activity

The effect of GLP-1 on the expression levels of GLUT2 and GK is shown in Fig. 2A. Pretreatment with GLP-1 for 60 min had no effect on GLUT2 and GK expression levels in INS-1 cells. To evaluate whether GLP-1 directly enhances GK activity, we measured GK activity in response to glucose (2–20 mM) with or without a 30-min GLP-1 pretreatment (Fig. 2B). As expected, GK activity was enhanced by GLP-1 when compared with the treatment without GLP-1. Although the V_{max} value for 100 nM GLP-1 was not significantly changed, the K_m value was decreased by a 30-min pretreatment with 100 nM GLP-1 (Table 1).

The GLP-1 effects in rat native β-cells

We further tested whether the effect of GLP-1 observed in INS-1 cells could also be observed in rat native β-cells (Figs. 3 and 4). Expectedly, GK activity (Fig. 3A), cellular ATP levels (Fig. 3B), and $\Delta\psi m$ (Fig. 4, A and B) measured in dispersed islet β-cells showed the same response as those in INS-1 cells. We also examined the effect of GLP-1 on oxygen consumption at different glucose concentrations in dispersed islet β-cells. Acute treatment with 100 nM GLP-1 further increased oxygen consumption in response to 7 mM glucose but not 15 mM glucose (Fig. 4C). After finishing all experiments, the dispersed islet cells were stained with insulin and glucagon antibodies to confirm purity of β-cells (data not shown).

GLP-1 enhances GK activity through cAMP and Epac

To investigate the signaling mechanism for the enhancement effect of GLP-1 on GK activity in INS-1 cells, experiments were performed with 7 mM glucose in the presence of various inhibitors of the GLP-1 signaling pathway. As shown in Fig. 5A, the effect of GLP-1 on GK activity was not observed in the presence of the adenylate cyclase inhibitor MDL-12330A (10 μM) or the nonselective Epac signaling inhibitor, brefeldin A (10 μg/ml) (27, 28). The following reagents were used to pharmacologically confirm the major involvement of cAMP/Epac in regulating GK activity in INS-1 β-cells without GLP-1: cAMP-elevating agent forskolin (100 μM), Epac-selective activator 8-pCPT-2′-*O*-Me-cAMP-AM (100 μM), and PKA-selective activator Sp-6-Bnz-cAMP (100 μM). The enhancing effects on GK activity most closely mimicking those of GLP-1 were obtained with forskolin and 8-pCPT-2′-*O*-Me-cAMP-AM (Fig. 5B).

GLP-1 promotes GK activity through Epac2, Rim2, and Rab3A

In β-cells, Epac2 is more abundant than Epac1 (29). Recently, Epac2, Rim2, and the Ca^{2+} sensor Piccolo have

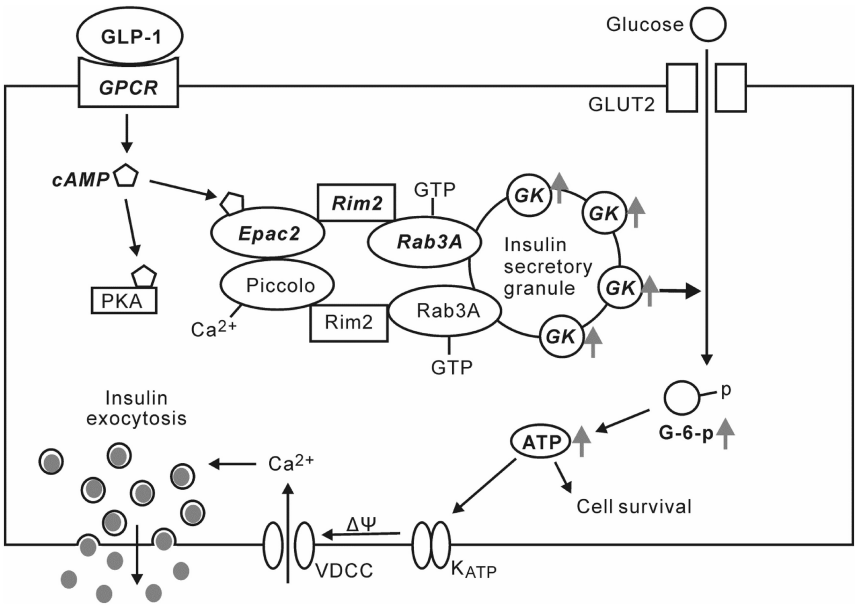

FIG. 7. Proposed mechanism to explain the effects of GLP-1 on GK activity in pancreatic β-cells. The cAMP-dependent interactions of Epac2, Rim2, and Piccolo based on an unknown stoichiometry are depicted (30). The Epac2/Rim2/Piccolo macromolecular complex interacts with the GTP-bound form of Rab3A to potentiate the activity of GK, which is an integral component of insulin secretory granules (31). Δψ, Membrane depolarization; VDCC, voltage-dependent calcium channels.

been reported to form a macromolecular complex in the presence of Ca^{2+}, which interacts with the GTP-bound form of Rab3A located on the cytoplasmic surface of insulin secretory granules to regulate priming and exocytosis (30). In addition, β-cell GK has been reported to be an integral component of insulin secretory granules (31). To confirm the association of the effect of GLP-1 on GK activity with the up-regulation of GLP-1 signaling, we suppressed the expression of Epac2, Rim2, or Rab3A in INS-1 cells using siRNA. As shown in Fig. 6A, the expression of each protein was reduced compared with the expression levels in control cells. As expected, the enhancement effects of GLP-1 on GK activity (Fig. 6B), 2-deoxyglucose uptake (Fig. 6C) and cellular ATP levels (Fig. 6D) were dramatically reduced in each protein-depleted cell. These data indicate that Epac2, Rim2, and Rab3A are all critical for the effects of GLP-1.

Discussion

In the present study, we demonstrated that 100 nM GLP-1 acutely enhanced GK activity in a glucose-dependent manner in rat INS-1 and native β-cells, which resulted in an increase in mitochondrial glucose metabolism, thus elevating cellular ATP levels. Accordingly, this might be one of the mechanisms of GLP-1 to facilitate glucose-mediated insulin secretion (Fig. 7). The rate of glucose transport in β-cells is usually determined by GK activity (32, 33), and small changes in β-cell GK activity have large effects on GSIS (34, 35). Although intracellular glucose appears to be the main endogenous GK activator in β-cells (36), the importance of GLP-1R signaling for GK function has already been implicated (7). Although the short-term treatment of GLP-1 in this study (60 min) did not increase GK expression, longer treatments with GLP-1 (24 h) does increase GK expression (21). Together, these findings suggest that both GK activity and GK expression are acting points of GLP-1R signaling. GLUT2 activation by GLP-1 may be another underlying mechanism for the enhancement effect of GLP-1 on cellular uptake of 2-deoxyglucose, which is a nonmetabolizable glucose that does not enter the glycolytic pathway despite its phosphorylation by GK. However, glucose uptake through the low-affinity glucose transporter, GLUT2, is usually not rate-limiting and is, therefore, unlikely to be the site for regulation of GLP-1 (37, 38). Rather, it has been suggested that GLP-1 negatively regulates GLUT2 activity in β-cells through PKA-dependent phosphorylation (39). GLUT2 activity has been shown to depend on the phosphorylations of the carboxyl-terminal tail (39). However, the rate of glucose transport in β-cells is 50- to 100-fold greater than the rate of GLUT2 phosphorylation. Moreover, a 70–80% reduction of GLUT2 expression is needed to impair GSIS (40). Thus, GLUT2 has mainly a permissive role allowing glucose unrestricted access to GK, which is the rate-limiting step of glucose metabolism in β-cells and may be an important target protein of GLP-1 signaling.

The present results suggested that the potentiated GK activity by 100 nM GLP-1 increased glucose uptake and mitochondrial glucose metabolism, thereby leading to more elevated cellular ATP levels in response to 7 mM glucose (approximately 126 mg/dl). Mukai *et al.* (41) recently found that the ATP content in islets of Goto-Kakizaki rats, which are characterized by an impaired high-glucose-induced increase in ATP production, is increased by 100 nM GLP-1 at a glucose concentration of 16.7 mM, and they reported that the ATP content in islets of Wistar rats, which are the normal control, is not changed by 100 nM GLP-1. This result may support the present data, which demonstrated no further enhancement of cellular ATP levels with 100 nM GLP-1 treatment at a glucose concentration of 15 mM.

Previous studies have clearly shown that GLP-1 facilitates not only GSIS but also β-cell survival through both

cAMP-dependent and cAMP-independent pathways by exploiting plasmalemmal GLP-1R and epidermal growth factor receptors (15). Cell survival and GSIS require adequate cellular response to glucose. In addition to previous studies, the potentiating action of GLP-1 on GK activity shown in the present study may also contribute to promoting β-cell survival and GSIS by enhancing cellular glucose sensitivity (22). GK with a low affinity for glucose is selectively expressed in glucose-sensing and hormone (transmitter)-secreting neuronal/neuroendocrine cells, gut endocrine cells, pancreatic β-cells, and hepatocytes (42). In a motor neuron cell line, we have recently found that GLP-1 selectively potentiates GK activity through cAMP/Epac signaling (43), which is a similar mechanism to the mechanism found in β-cells in the present study. In contrast, acute glucose uptake regulation by GLP-1 is likely to be mediated by GLUT4 trafficking to the plasma membrane via phosphoinositide 3-kinase and/or protein kinase B in skeletal muscles and adipocytes that possess only the other high-affinity hexokinases for glucose (44, 45).

The action of GLP-1 on β-cell that activates adenylate cyclase (46) to increase cytosolic cAMP levels is generally thought to activate the downstream signaling of PKA (15, 20, 47) and Epac (19, 25, 48). The present results suggested that cAMP/Epac signaling was more critical than cAMP/PKA signaling for GK activation by GLP-1. Epac proteins are novel cAMP sensors that regulate several pivotal cellular processes (49–52). Although two variants of Epac (Epac1 and Epac2) are expressed in β-cells, Epac2 mRNA levels are approximately 125-fold higher than Epac1 mRNA levels in β-cells (29). Thus, Epac2 most likely constitutes the predominant effector protein activated by GLP-1 in β-cells. For Epac2-mediated enhancement of insulin exocytosis, it has been recently reported that Rim2 and the Ca^{2+} sensor Piccolo form a macromolecular complex with Epac2 in the presence of Ca^{2+}, but the stoichiometry of this complex has yet to be determined (30). This complex then interacts with the GTP-bound form of Rab3A located on the cytoplasmic surface of insulin secretory granules. Although some GK can be found in the mitochondrial-rich fraction, the majority of GK has been reported to be an integral component of insulin secretory granules (31) where Rab3A is embedded. The present findings support that both Rim2 and Rab3A are needed for Epac2 to enhance GK activity at least in INS-1 cells. Therefore, it may be suggested that GLP-1 could stimulate both GK activity and insulin exocytosis with a common mechanism via association of Epac2 with Rim2 and Rab3A downstream of GLP-1R and cAMP. By activating GK, GLP-1 facilitates the glucose supply available for mitochondrial glucose metabolism. In addition, GK associated with insulin granules may also participate in glucose-induced changes in granule movement or membrane fusion during insulin secretion (31). The GLP-1-mediated modulation of GK activity is glucose dependent, which is similar to the other roles of GLP-1 in GSIS, and the GLP-1-mediated modulation of GK activity is not observed at low glucose concentrations, which is a finding relevant to clinical applications in treating T2D.

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