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Role of Epac2A/Rap1 Signaling in Interplay Between Incretin and Sulfonylurea in Insulin Secretion

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Incretin-related drugs and sulfonylureas are currently used worldwide for the treatment of type 2 diabetes. We recently found that Epac2A, a cAMP binding protein having guanine nucleotide exchange activity toward Rap, is a target of both incretin and sulfonylurea. This suggests the possibility of interplay between incretin and sulfonylurea through Epac2A/Rap1 signaling in insulin secretion. In this study, we examined the combinatorial effects of incretin and various sulfonylureas on insulin secretion and activation of Epac2A/Rap1 signaling. A strong augmentation of insulin secretion by combination of GLP-1 and glibenclamide or glimepiride, which was found in *Epac2A*$^{+/+}$ mice, was markedly reduced in *Epac2A*$^{-/-}$ mice. In contrast, the combinatorial effect of GLP-1 and gliclazide was rather mild, and the effect was not altered by Epac2A ablation. Activation of Rap1 was enhanced by the combination of an Epac-selective cAMP analog with glibenclamide or glimepiride but not gliclazide. In diet-induced obese mice, ablation of Epac2A reduced the insulin secretory response to coadministration of the GLP-1 receptor agonist liraglutide and glimepiride. These findings clarify the critical role of Epac2A/Rap1 signaling in the augmenting effect of incretin and sulfonylurea on insulin secretion and provide the basis for the effects of combination therapies of incretin-related drugs and sulfonylureas.

ISLET STUDIES

Recently developed incretin-related drugs such as dipeptidyl peptidase-4 (DPP-4) inhibitors and GLP-1 receptor agonists are increasingly being used worldwide in the treatment of type 2 diabetes. Incretins such as GLP-1 and glucose-dependent insulinotropic polypeptide, which are secreted from the intestine upon meal ingestion, amplify insulin secretion from pancreatic β-cells in a glucose concentration–dependent manner (1,2). This glucose dependency of incretin action provided the basis for recently developed incretin-based therapies (3), which have less risk for hypoglycemia. GLP-1 and glucose-dependent insulinotropic polypeptide bind to their specific receptors on pancreatic β-cells, increasing the intracellular cAMP level through the activation of adenylate cyclase, thereby leading to the potentiation of insulin secretion. This potentiation is mediated by both protein kinase A (PKA)-dependent and PKA-independent pathways, the latter involving Epac2, a protein possessing guanine nucleotide exchange activity toward the small GTPase Rap (4–6). Three subtypes of Epac2 have been identified, as follows: Epac2A (originally referred to as Epac2), mainly expressed in the brain and neuroendocrine and endocrine tissues; Epac2B, in the adrenal gland (7); and Epac2C, in the liver (8). Epac2A/Rap1 signaling has been shown to be required for the potentiation of the first phase of glucose-induced insulin secretion in the pancreatic β-cell (9–11).

On the other hand, sulfonylureas are antidiabetic drugs commonly used for many years. A primary target of sulfonylureas is the ATP-sensitive potassium (K_{ATP})

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channels in pancreatic β-cells. Binding of sulfonylureas to SUR1, the regulatory subunit of the K_{ATP} channel, causes closure of the channel, resulting in depolarization of the β-cells and opening of voltage-dependent Ca^{2+} channels (VDCCs). The influx of extracellular Ca^{2+} through VDCC triggers insulin secretion (12–14).

We have previously found that Epac2A is also a direct target of sulfonylureas and that activation of Epac2A/Rap1 signaling is required for sulfonylurea-induced insulin secretion (15). Thus, Epac2A is a target of both incretin/cAMP signaling and sulfonylureas. We recently identified the sulfonylurea binding site in Epac2A and characterized the binding properties of various sulfonylureas to Epac2A (16), and found that cAMP signaling and sulfonylurea cooperatively activate Epac2A.

In clinical settings, combination therapies of incretin-related drugs and sulfonylureas are often used for glycemic control in type 2 diabetes (17,18) but lead to hypoglycemia in some cases (19). Coadministration of incretin and sulfonylurea has been found to enhance insulin secretion in humans (20,21). However, the underlying mechanism for the augmentation of insulin secretion by the combination of incretin/cAMP signaling and sulfonylurea is not known. In the current study, we have examined the role of Epac2A/Rap1 signaling in the interplay between incretin/cAMP signaling and sulfonylurea in insulin secretion.

RESEARCH DESIGN AND METHODS

Animals and Diet

Epac2A$^{-/-}$ mice were generated as previously described (9). *Epac2A*$^{+/-}$ mice with C57BL/6 background were maintained to obtain *Epac2A*$^{+/+}$ and *Epac2A*$^{-/-}$ littermates. For a high-fat diet (HFD) study, mice were fed with an HFD (Research Diets, Inc., New Brunswick, NJ) for 6 weeks from 4 or 5 weeks of age. All animal experiments were performed in accordance with the guidelines of the Kobe University Animal Ethics Committee of Kobe University Graduate School of Medicine. HFD mice experiments were approved (KM-2013–42) by the Keimyung University Institutional Ethics Committee.

Reagents

Glibenclamide and gliclazide were purchased from Sigma-Aldrich (St. Louis, MO). Glimepiride was from Wako (Osaka, Japan). 6-Bnz-cAMP-AM (6-Bnz), 8-pCPT-2′-*O*-Me-cAMP-AM (8-pCPT), and 8-Br-cAMP-AM were from BIOLOG Life Science Institute (Bremen, Germany). GLP-1 was from The Peptide Institute (Osaka, Japan). Anti-CREB antibody and anti–phospho-CREB antibody were purchased from Cell Signaling Technology (Danvers, MA).

Insulin Secretion Experiments

Pancreatic islets were isolated from C57BL/6 mice by collagenase digestion and cultured for 2 days, as described previously (6). Thirty minutes after preincubation of isolated islets with Krebs-Ringer bicarbonate HEPES buffer (KRBH) containing 2.8 mmol/L glucose, five size-matched islets were collected in each well of a 96-well plate and incubated for 30 min in 100 μL of the same buffer containing various stimuli. To assess insulin content, the islets were extracted in acid ethanol overnight at 4°C. Insulin released in the incubation buffer and islet insulin contents were measured by homogeneous time-resolved fluorescence assay using an insulin assay kit (CIS Bio International, Gif-sur-Yvette, France). The amount of insulin secretion was normalized by islet insulin content.

Perfusion Experiments

Perfusion experiments were performed as previously described (22). Briefly, overnight (16 h) fasted male mice 16–25 weeks of age were used. The perfusion protocol began with a 20-min equilibration period with the same buffer used in the initial step shown in the figures. The flow rate of the perfusate was 1 mL/min. The insulin levels in the perfusate were measured by homogeneous time-resolved fluorescence assay.

Cell Culture

MIN6-K8 cells were grown in DMEM (Sigma-Aldrich) containing 10% heat-inactivated FBS and maintained in a humidified incubator with 95% air and 5% CO_2 at 37°C (23).

Measurement of Rap1 Activity

Pull-down assay for Rap1-GTP (guanosine triphosphate) was performed as described previously (9). DMSO was used as a vehicle. Precise quantification was achieved by densitometric analysis of the immunoreactive bands with the National Institutes of Health ImageJ software. The intensity of the Rap1-GTP signal was normalized to that of total Rap1. Anti-Rap1 antibody was purchased from Millipore (Bedford, MA).

Small Interfering RNA Knockdown Studies

For Rap1a and Rap1b knockdown experiments, small interfering RNAs (siRNAs) (siGENOME SMARTpool and ON-TARGETplus nontargeting pool) were purchased from Dharmacon (Lafayette, CO). MIN6-K8 cells were transfected with siRNAs using DharmaFECT2 transfection reagent (Dharmacon) according to the manufacturer's instructions.

Measurement of Intracellular Ca^{2+} Concentration

Primary cultured β-cells isolated from mouse pancreatic islets were loaded with 2 μmol/L Fura Red-AM (Invitrogen) for 30 min at 37°C in KRBH. The cells were stimulated with indicated secretagogues and excited at 480/440 nm using an Olympus IX-71 microscope coupled to an ImagEM camera (Hamamatsu Photonics, Hamamatsu, Japan). The images were acquired by MetaMorph (Molecular Devices, CA).

Total Internal Reflection Fluorescence Microscopy

Primary cultured β-cells isolated from mouse pancreatic islets were infected with adenovirus carrying insulin-Venus and subjected to analysis by total internal reflection fluorescence microscopy (TIRFM) as previously described (9). Cells were preincubated in KRBH containing

4.4 mmol/L glucose for 30 min, and then various agents were added to the chamber at the final concentrations that are indicated in the figures. Images were acquired every 250 ms by MetaMorph.

In Vivo Experiments

For liraglutide and glimepiride challenge test, after 12-h fasting, mice were administered with liraglutide (6.0 mg/mL; Victoza; Novo Nordisk) (300 μg/kg i.p.) and glimepiride (1 mg/kg orally via gavage). For oral glucose tolerance testing, liraglutide (300 μg/kg i.p.) and glimepiride (1 mg/kg orally via gavage) were administered after 16 h of fasting to mice 15 min before glucose (1.5 g/kg) loading. Serum insulin levels were measured using Mouse Insulin ELISA Kit (Morinaga Institute of Biological Science, Inc., Yokohama, Japan) and Ultrasensitive Mouse Insulin ELISA (Mercodia, Uppsala, Sweden).

Statistical Analysis

The data are expressed as means ± SEM. Comparisons were made using Student unpaired *t* test, Dunnett test, or Tukey-Kramer test, as indicated in the legends. A probability level of $P < 0.05$ was considered statistically significant.

RESULTS

Combination of GLP-1 and Sulfonylurea Augments Insulin Secretion From Mouse Pancreatic Islets

The combinatorial effects of GLP-1 and glibenclamide, glimepiride, or gliclazide on insulin secretion from mouse pancreatic islets were first examined in the presence of 8.8 mmol/L glucose, a glucose-stimulated condition (Fig. 1*A*). Glucose-induced insulin secretion was augmented by 100 nmol/L glibenclamide and 10 nmol/L GLP-1. The combination of GLP-1 and glibenclamide exhibited an additive effect on insulin secretion. Similar effects were observed by the combination of GLP-1 and glimepiride or gliclazide. At a basal level of glucose (4.4 mmol/L glucose), 10 nmol/L GLP-1 alone did not induce insulin secretion, but synergistically augmented glibenclamide-induced insulin secretion (Fig. 1*B*). A similar synergistic effect was observed by the combination of GLP-1 with glimepiride or gliclazide.

Augmentation by GLP-1 of Glibenclamide- or Glimepiride-Induced Insulin Secretion, but Not Gliclazide-Induced Insulin Secretion, Is Reduced in *Epac2A*$^{-/-}$ Mice

To examine the augmenting effect of GLP-1 and sulfonylurea on insulin secretion at the basal level of glucose concentration in detail, we investigated the dynamics of insulin secretion by perfusion of mouse pancreas. In *Epac2A*$^{+/+}$ mice, 100 nmol/L glibenclamide induced insulin secretion in a biphasic manner: a transient increase immediately after stimulation (first phase), followed by sustained release (second phase) at 4.4 mmol/L glucose (Fig. 2*A*, left). The first phase of insulin secretion induced by glibenclamide tended to be reduced in *Epac2A*$^{-/-}$ mice. At 4.4 mmol/L glucose, 10 nmol/L GLP-1 alone did not

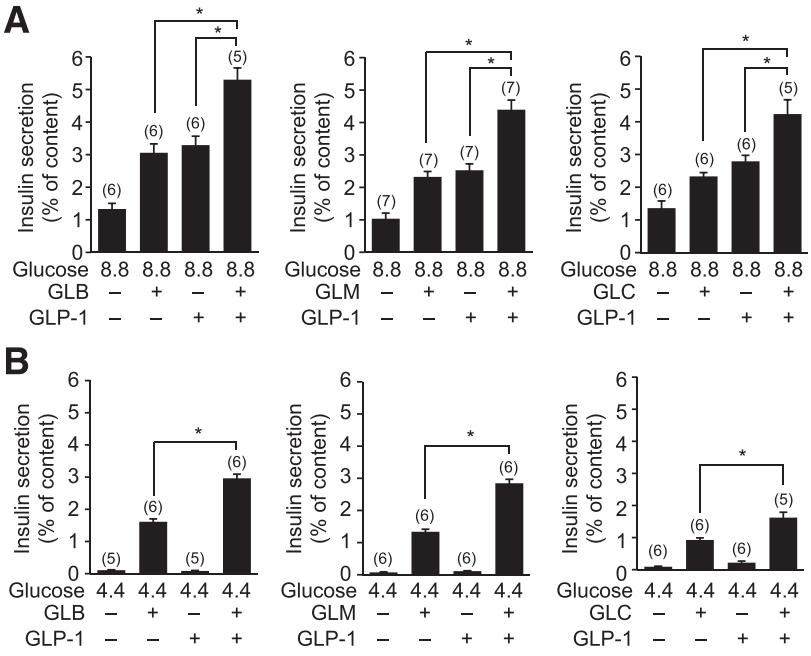

Figure 1—Combination of GLP-1 and sulfonylureas augments insulin secretion from mouse pancreatic islets. *A*: Insulin secretion from mouse pancreatic islets stimulated with 10 nmol/L GLP-1 and 100 nmol/L glibenclamide (GLB) (left), 100 nmol/L glimepiride (GLM) (middle), or 5 μmol/L gliclazide (GLC) (right) at 8.8 mmol/L glucose for 30 min. *B*: Insulin secretion from mouse pancreatic islets stimulated with 10 nmol/L GLP-1 and 100 nmol/L GLB (left), 100 nmol/L GLM (middle), or 5 μmol/L GLC (right) at 4.4 mmol/L glucose for 30 min. Data are expressed as means ± SEM. The numbers of wells are indicated above the columns. The representative data of three independent experiments are shown. *$P < 0.01$ (Tukey-Kramer test).

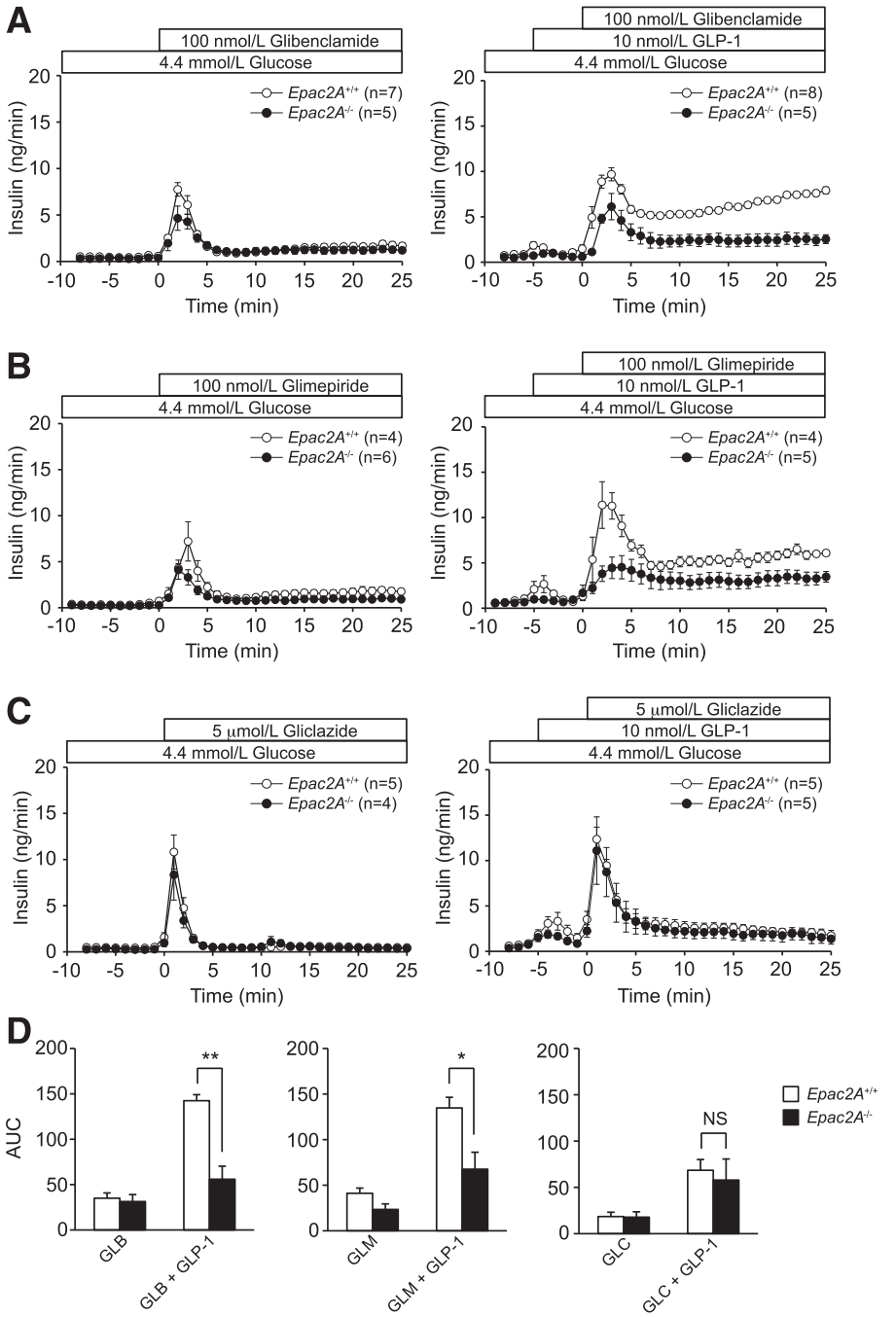

Figure 2— Augmentation by GLP-1 of glibenclamide (GLB)- and glimepiride (GLM)-induced, but not gliclazide (GLC)-induced, insulin secretion is reduced in *Epac2A*$^{-/-}$ mice. *A–C*: Comparison between *Epac2A*$^{+/+}$ and *Epac2A*$^{-/-}$ mice of insulin secretion from perfused mouse pancreata stimulated with 100 nmol/L GLB alone (*A*, left); 100 nmol/L GLM alone (*B*, left); 5 μmol/L GLC alone (*C*, left); and combination of 10 nmol/L GLP-1 with 100 nmol/L GLB (*A*, right), 100 nmol/L GLM (*B*, right), or 5 μmol/L GLC (*C*, right) at 4.4 mmol/L glucose. *D*: Comparison between *Epac2A*$^{+/+}$ and *Epac2A*$^{-/-}$ mice of the AUC for insulin secretion induced by each stimulus indicated. Data are expressed as means ± SEM of four to eight mice for each group. $^{*}P < 0.05$, $^{**}P < 0.01$ (Student unpaired *t* test). NS, not significant.

induce insulin secretion in either *Epac2A*$^{+/+}$ or *Epac2A*$^{-/-}$ mice (Supplementary Fig. 1). The combination of GLP-1 and glibenclamide synergistically augmented insulin secretion in both the first and second phases of insulin secretion in *Epac2A*$^{+/+}$ mice, while the augmenting effect on both phases was markedly reduced in *Epac2A*$^{-/-}$ mice (Fig. 2*A*, right). We have recently found that, like glibenclamide, glimepiride activates Epac2A, as assessed by Epac2A fluorescence resonance energy transfer sensor (16). Glimepiride also activated Rap1 in a dose-dependent manner in MIN6-K8 cells (Supplementary Fig. 2). The first phase of glimepiride-induced insulin secretion tended to be reduced (Fig. 2*B*, left), and the augmenting effect of GLP-1 and glimepiride on insulin secretion was markedly

reduced (Fig. 2*B*, right) in *Epac2A*$^{-/-}$ mice. Gliclazide induced a transient insulin secretion immediately after stimulation, and the dynamics of insulin secretion in *Epac2A*$^{-/-}$ mice were nearly identical to those in *Epac2A*$^{+/+}$ mice (Fig. 2*C*, left). GLP-1 moderately augmented gliclazide-induced insulin secretion, predominantly in the second phase (Fig. 2*C*, right). There was no significant difference between *Epac2A*$^{+/+}$ and *Epac2A*$^{-/-}$ mice in the augmentation by GLP-1 of gliclazide-induced insulin secretion. The area under the curve (AUC) calculated for minutes 0–25 (sulfonylurea stimulation) showed a significant reduction in the augmentation by the combination of GLP-1 with glibenclamide and glimepiride, but not with gliclazide, in *Epac2A*$^{-/-}$ mice (Fig. 2*D*).

Epac2A Plays a Major Role in the Augmentation of Glibenclamide-Induced Insulin Secretion by cAMP Signaling

To determine the roles of PKA and Epac2A in the augmentation by GLP-1 of glibenclamide-induced insulin secretion, we used 6-Bnz and 8-pCPT, cAMP analogs that are specific for PKA and Epac, respectively. The effects of 6-Bnz and 8-pCPT on the phosphorylation of CREB and the activation of Rap1 in MIN6-K8 cells were examined. 6-Bnz significantly increased the level of phosphorylated CREB, whereas 8-pCPT did not increase them at the concentrations used (Supplementary Fig. 3*A*). Rap1 was significantly activated by 5 and 10 μmol/L 8-pCPT, but not by 6-Bnz at any concentration used (Supplementary Fig. 3*B* and *C*). 6-Bnz exhibited little effect on glibenclamide-induced insulin secretion at 10 μmol/L and augmented it predominantly in the second phase at 30 μmol/L (Fig. 3*A*, left, and *B*); whereas, 8-pCPT at both 5 and 10 μmol/L markedly augmented glibenclamide-induced insulin secretion in both the first and second phases (Fig. 3*A*, right, and *B*), indicating that Epac2A rather than PKA plays a major role in the augmentation of sulfonylurea-induced insulin secretion by cAMP signaling.

Rap1 Activation Is Markedly Enhanced by Combination of an Epac-Selective cAMP Analog With Glibenclamide or Glimepiride, but Not With Gliclazide

The combinatorial effect of a cAMP analog and sulfonylurea on the activation of Rap1, the downstream signaling of Epac2A, was examined using MIN6-K8 cells. A significant enhancement of Rap1 activation was found by costimulation of glibenclamide and 8-pCPT at 5 or 10 μmol/L (Fig. 4*A*). In contrast, Rap1 activation by glibenclamide was not affected by the combination of glibenclamide with 6-Bnz at concentrations that sufficiently phosphorylate CREB (Fig. 4*B*). The combination of glimepiride and 10 μmol/L 8-pCPT markedly enhanced Rap1 activation (Fig. 4*C*), whereas the combination of

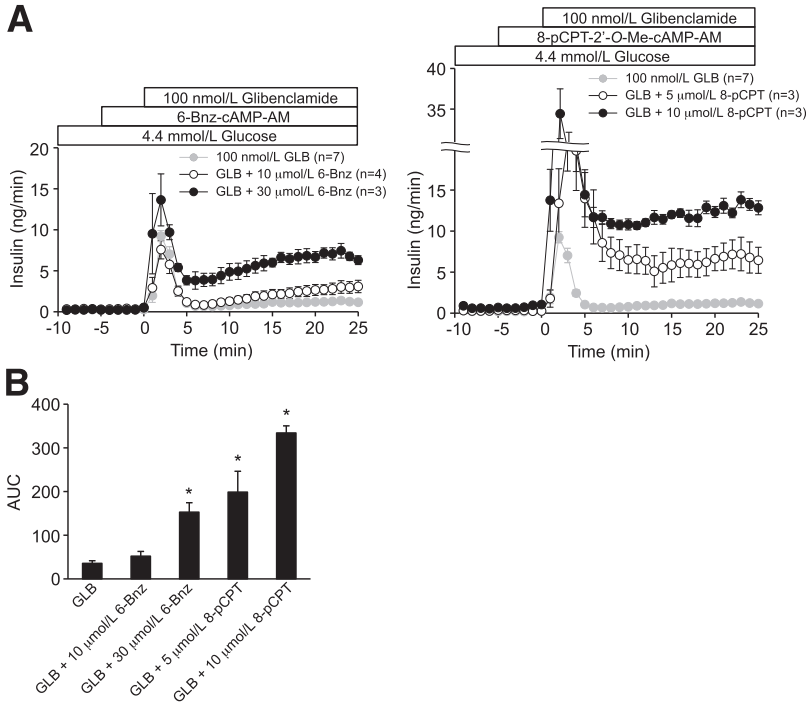

Figure 3—Epac2A plays a major role in the augmentation of glibenclamide (GLB)-induced insulin secretion by cAMP signaling. *A*: Effects of 6-Bnz, a PKA-selective cAMP analog, at 10 μmol/L (open circles) and 30 μmol/L (closed circles) on the dynamics of insulin secretion from mouse perfused pancreata induced by 100 nmol/L GLB at 4.4 mmol/L glucose (left), and effects of 8-pCPT at 5 μmol/L (open circles) and 10 μmol/L (closed circles) on the dynamics of insulin secretion from mouse perfused pancreata induced by 100 nmol/L GLB at 4.4 mmol/L glucose (right). Gray circles represent insulin secretion induced by 100 nmol/L GLB alone (*A*). *B*: Comparison of AUC for insulin secretion induced by each stimulus indicated. Data are expressed as means ± SEM. *$P < 0.01$ (Dunnett test).

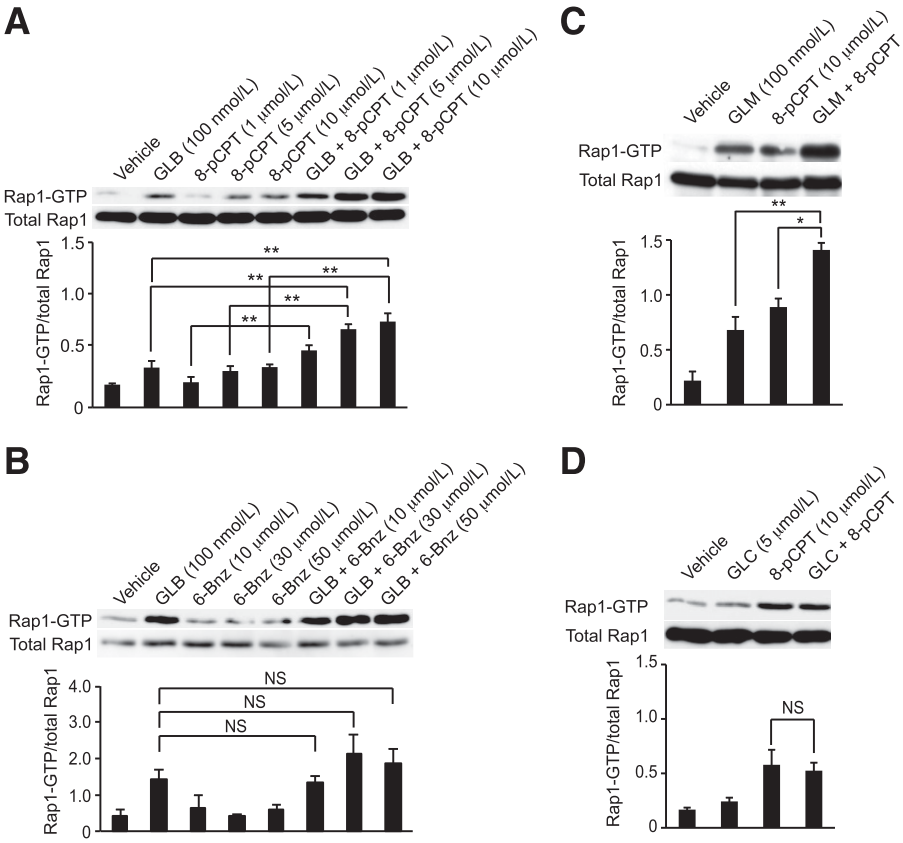

Figure 4— Rap1 activation is markedly enhanced by the combination of 8-pCPT with glibenclamide (GLB) or glimepiride (GLM), but not with gliclazide (GLC). *A*: Dose-dependent effects of 8-pCPT on the enhancement of Rap1 activation in MIN6-K8 cells. Cells were treated with each stimulus indicated for 15 min. Data are expressed as means ± SEM of four independent experiments. *B*: Combinatorial effects of 100 nmol/L GLB with 10, 30, or 50 μmol/L 6-Bnz. Data are expressed as means ± SEM of four independent experiments. *C*: Combinatorial effects of 100 nmol/L GLM with 10 μmol/L 8-pCPT. Data are expressed as means ± SEM of four independent experiments. *D*: Combinatorial effects of 5 μmol/L GLC with 10 μmol/L 8-pCPT. Data are expressed as means ± SEM of four independent experiments. $^{*}P < 0.01$, $^{**}P < 0.001$ (Tukey-Kramer test). NS, not significant.

gliclazide and 8-pCPT did not (Fig. 4*D*). These results indicate that the combination of GLP-1 with glibenclamide or glimepiride, but not with gliclazide, enhances Epac2A/Rap1 signaling and that PKA activation is not involved in Epac2A/Rap1 activation.

Activation of Rap1 Is Required for the Augmenting Effect of Incretin/cAMP Signaling and Glibenclamide on Insulin Secretion

To determine the role of the enhanced Rap1 activation in insulin secretion, we used Epac2A(465–1011), a constitutive active mutant of Epac2A lacking both cyclic nucleotide-binding domain (cNBD) A and cNBD-B (Fig. 5*A*). X-ray crystallographic analysis of Epac2A revealed that the access of Rap1 to the catalytic region of Epac2A is sterically blocked by the regulatory region including two cNBDs in the absence of cAMP (24). The binding of cAMP to Epac2A induces a conformational change, releasing a catalytic region that leads to Rap1 binding and activation (25,26). The catalytic region of Epac2A(465–1011) is open for the access of Rap1, thus constitutively exhibiting guanine nucleotide exchange activity toward Rap1. In the MIN6-K8 cells infected with adenovirus carrying Epac2A(465–1011), Rap1 was strongly activated in the absence of cAMP (Fig. 5*B*). The infection of MIN6-K8 cells with adenovirus carrying Epac2A(465–1011), but not full-length Epac2A, augmented glibenclamide-induced insulin secretion (Fig. 5*C* and Supplementary Fig. 4). The augmenting effect of introducing Epac2A(465–1011) on insulin secretion was dependent on the dose of adenovirus. The introduction of Epac2A(465–1011) into MIN6-K8 cells also augmented glucose-induced insulin secretion significantly (Supplementary Fig. 5). In contrast, knockdown of Rap1 expression by siRNA in MIN6-K8 cells significantly reduced the augmentation by GLP-1 or 8-pCPT of glibenclamide-induced insulin secretion (Fig. 5*D*). These results indicate that Rap1 activation is required for the augmenting effects of incretin and sulfonylureas on insulin secretion.

Costimulation by Glibenclamide and GLP-1 Augments the Rise in Ca^{2+} Level in Primary Cultured β-Cells of *Epac2A*$^{+/+}$ Mice, but Not *Epac2A*$^{-/-}$ Mice

Since Epac2A/Rap1 signaling has been shown to modulate the intracellular Ca^{2+} level in pancreatic β-cells (27), we next examined the combinatorial effects of GLP-1 and

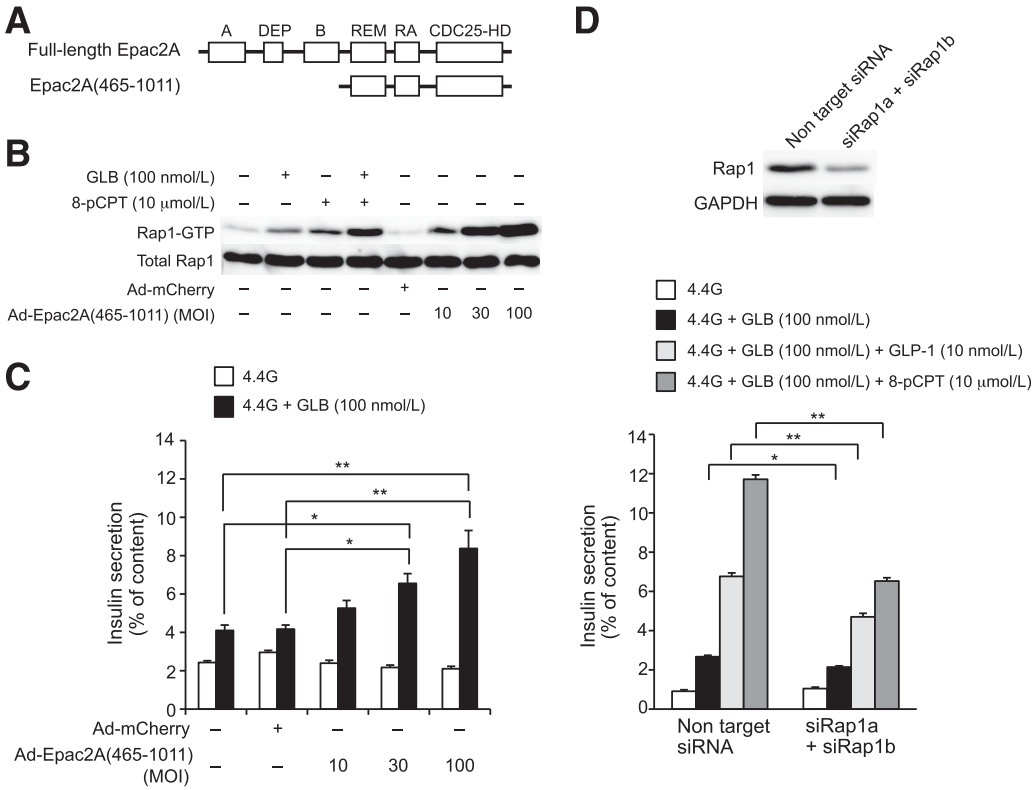

Figure 5—Activation of Rap1 is required for the augmenting effect of GLP-1 or 8-pCPT and glibenclamide (GLB) on insulin secretion. *A*: Domain structures of full-length Epac2A and Epac2A(465–1011). A, cNBD-A; DEP, Dishevelled, Egl-10, and Pleckstrin domain; B, cNBD-B; REM, Ras exchange motif; RA, Ras association domain; CDC25-HD, CDC25 homology domain. *B*: Rap1 activation in noninfected MIN6-K8 cells or MIN6-K8 cells infected with adenovirus carrying mCherry or Epac2A(465–1011) at a multiplicity of infection (MOI) of 10, 30, or 100. *C*: Insulin secretion from noninfected MIN6-K8 cells or MIN6-K8 cells infected with adenovirus carrying mCherry or Epac2A(465–1011) at an MOI of 10, 30, or 100. Data are expressed as means ± SEM (n = 4 for each group). $^*P < 0.05$, $^{**}P < 0.01$ (Tukey-Kramer test). *D*: Effects of Rap1 knockdown on insulin secretion from MIN6-K8 cells. Top: Efficiency of knockdown was confirmed by immunoblot analysis. Bottom: Insulin secretion from MIN6-K8 cells transfected with nontarget siRNA or siRNA for Rap1a and Rap1b. Data are expressed as means ± SEM (n = 4 for each group). $^*P < 0.005$, $^{**}P < 0.001$ (Tukey-Kramer test).

glibenclamide on intracellular Ca^{2+} level in primary cultured β-cells from *Epac2A*$^{+/+}$ mice and *Epac2A*$^{-/-}$ mice. In *Epac2A*$^{+/+}$ β-cells, costimulation by 10 nmol/L GLP-1 and 100 nmol/L glibenclamide induced a rise in Ca^{2+} level greater than that by stimulation with 100 nmol/L glibenclamide alone at 4.4 mmol/L glucose (Fig. 6*A* and *B*). In contrast, in *Epac2A*$^{-/-}$ β-cells, the augmentation of Ca^{2+} level by the combination of GLP-1 and glibenclamide was diminished (Fig. 6*C* and *D*). These results indicate that the intracellular Ca^{2+} level is involved in the augmentation of glibenclamide-induced insulin secretion by GLP-1 at 4.4 mmol/L glucose.

Combination of GLP-1 and Glimepiride Markedly Enhances Insulin Granule Exocytosis

To examine the combinatorial effects of GLP-1 and glimepiride on insulin granule dynamics, we performed TIRFM analysis (9). In the presence of 4.4 mmol/L glucose, glimepiride at 100 nmol/L induced insulin granule exocytosis in a biphasic manner, dynamics that are similar to those of insulin secretion by glimepiride from perfused pancreata (Fig. 7*A*, left, and Supplementary Movie 1). A great number of fusion events were observed immediately after stimulation, and both the first and the second phases in insulin granule exocytosis were significantly augmented by the combination of GLP-1 and glimepiride (Fig. 7*A*, right, and Supplementary Movie 2). The combination of GLP-1 and glimepiride increased the number of fusion events derived from granules that are newly recruited, docked, and fused to the plasma membrane by stimulation (*resting newcomer*) as well as granules that are newly recruited and immediately fused to the plasma membrane by stimulation (*restless newcomer*) (9) (Fig. 7*B*).

Insulin Secretory Response to Coadministration of Liraglutide and Glimepiride Is Reduced in *Epac2A*$^{-/-}$ Mice With Diet-Induced Obesity

We next examined whether the combinatorial effect of incretin and sulfonylurea on blood glucose and insulin levels is mediated by Epac2A in vivo. Concomitant administration of liraglutide, a GLP-1 receptor agonist, and glimepiride lowered the blood glucose level by ~30% compared with the level before administration and

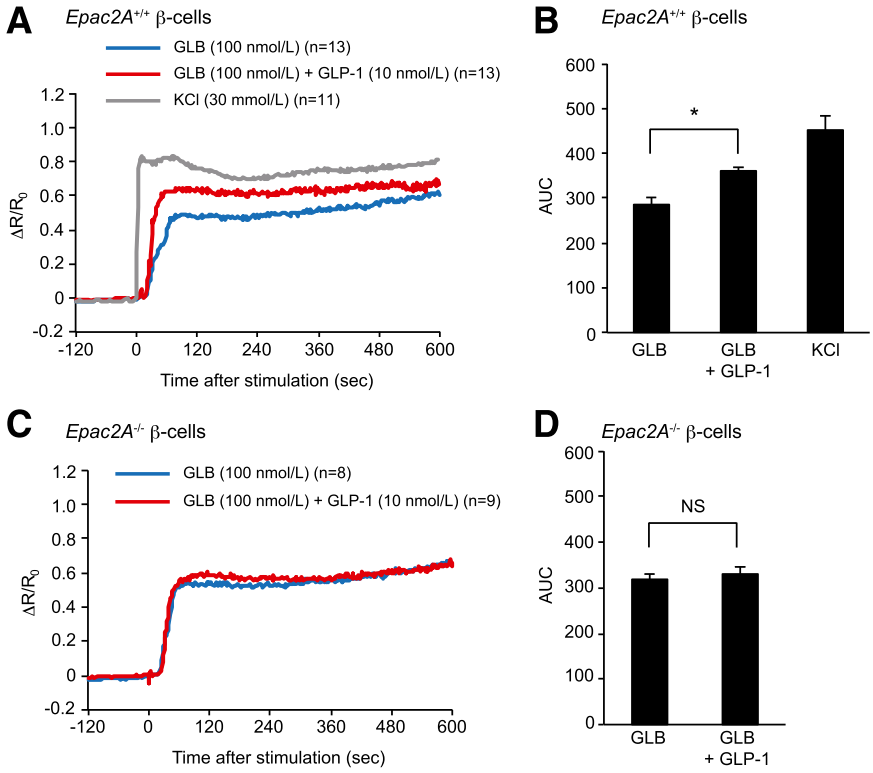

Figure 6—Costimulation of glibenclamide (GLB) and GLP-1 augments the rise in Ca^{2+} level in primary cultured β-cells of *Epac2A*$^{+/+}$ mice, but not *Epac2A*$^{-/-}$ mice. Change in intracellular Ca^{2+} concentration induced by 100 nmol/L GLB alone (blue line) and the combination of 100 nmol/L GLB and 10 nmol/L GLP-1 (red line) at 4.4 mmol/L glucose in primary cultured β-cells from *Epac2A*$^{+/+}$ (*A*) and *Epac2A*$^{-/-}$ (*C*) mice. Gray line indicates change in Ca^{2+} concentration induced by 30 mmol/L KCl in *Epac2A*$^{+/+}$ β-cells. Traces are mean values for the indicated number of β-cells. Data are expressed as the change in the ratio (ΔR) of the fluorescence emission at 440/480 nm, normalized to the ratio at 0 s (R_0) for the same cell. Comparison of the AUC for the 10-min period after stimulation in *Epac2A*$^{+/+}$ (*B*) and *Epac2A*$^{-/-}$ (*D*) β-cells. Data are expressed as means ± SEM. *$P < 0.05$ (Tukey-Kramer test). NS, not significant.

induced insulin secretion in *Epac2A*$^{+/+}$ mice (Supplementary Fig. 6). In *Epac2A*$^{-/-}$ mice, although the blood glucose level was lowered to almost the same extent as that of *Epac2A*$^{+/+}$ mice by administration of the drugs, the insulin secretory response tended to be reduced. It has recently been reported (28) that the insulin secretory response to intraperitoneal glucose load was impaired in *Epac2A*$^{-/-}$ mice with diet-induced obesity. We therefore examined the combinatorial effect of liraglutide and glimepiride on the mice that were fed an HFD. The blood glucose levels in *Epac2A*$^{-/-}$ mice were higher than those in *Epac2A*$^{+/+}$ mice during the experiment (Fig. 8*A*, left). Although the fasting serum insulin level of *Epac2A*$^{-/-}$ mice was higher than that of *Epac2A*$^{+/+}$ mice, the combinatorial effect on the insulin secretory response was almost completely abolished in *Epac2A*$^{-/-}$ mice (Fig. 8*A*, right). We then examined the combinatorial effect of liraglutide and glimepiride on blood glucose levels and insulin levels after oral glucose loading. *Epac2A*$^{-/-}$ mice exhibited higher blood glucose levels and reduced insulin response compared with *Epac2A*$^{+/+}$ mice (Fig. 8*B*). These results indicate that the glucose-lowering effect of the combination of liraglutide and glimepiride is diminished in *Epac2A*$^{-/-}$ mice. Thus, Epac2A plays a critical role in insulin secretion induced by the combination of incretin and sulfonylurea, especially in a model of diet-induced obesity.

DISCUSSION

We have previously shown that Epac2A is a target of both incretin/cAMP signaling and sulfonylurea in insulin secretion (5,6,15). The current study shows that incretin and the sulfonylureas glibenclamide and glimepiride synergistically stimulate insulin secretion at a basal level of glucose concentration (4.4 mmol/L) through Epac2A/Rap1 signaling. Our data also indicate that the synergistic effect of GLP-1 and gliclazide is rather mild and is not mediated by Epac2A/Rap1 signaling. These results are supported by our recent findings that cAMP and sulfonylurea (except for gliclazide) cooperatively activate Epac2A (16). Gliclazide is unique among sulfonylureas in that its effect is not influenced by Epac2A/Rap1 signaling. The differences in the action of various sulfonylureas on Epac2A may well account for the differences in the combinatorial effects of incretin and sulfonylureas.

Epac2A is known to regulate various cellular functions through the activation of Rap1 (29–31). In pancreatic β-cells, Rap1 mediates Epac2A-dependent amplification of insulin secretion (9). Rap1 has recently been shown

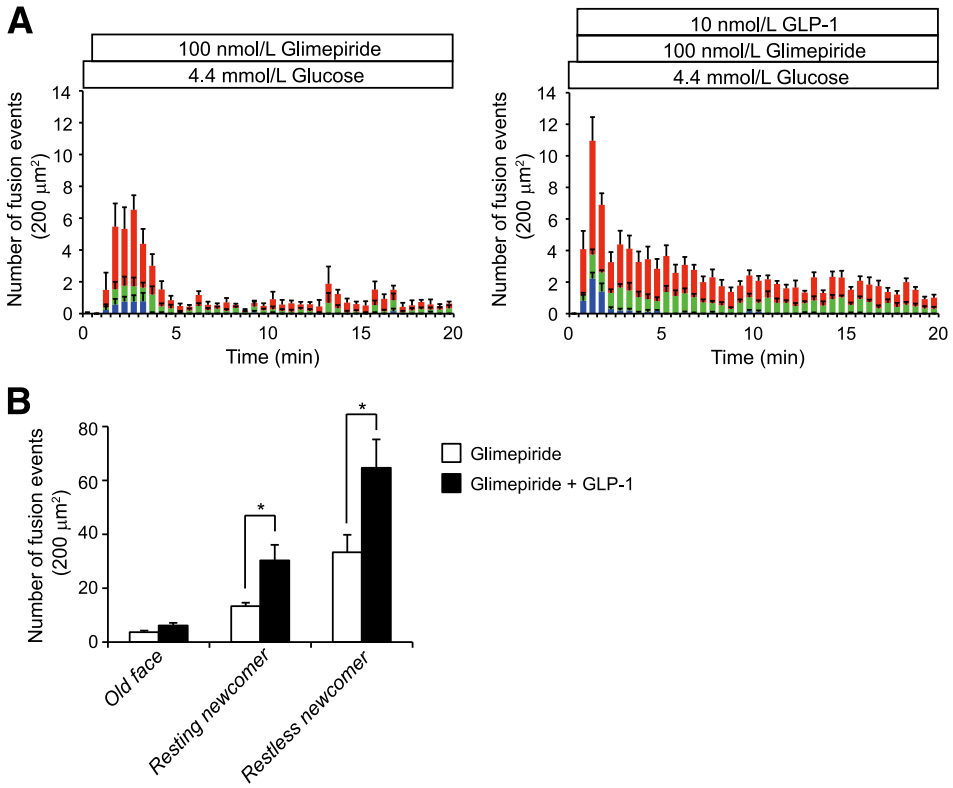

Figure 7—Combination of GLP-1 and glimepiride markedly enhances insulin granule exocytosis. *A*: Histogram of fusion events at 30 s intervals in primary cultured pancreatic β-cells. Cells were stimulated with 100 nmol/L glimepiride alone (left) and a combination of 100 nmol/L glimepiride and 10 nmol/L GLP-1 (right) at 30 s (see Supplementary Movie 1 [100 nmol/L glimepiride] and Supplementary Movie 2 [100 nmol/L glimepiride and 10 nmol/L GLP-1]). *Old face* (blue), granules that are predocked to the plasma membrane and fused to the membrane by stimulation; *Restless newcomer* (red), granules that are newly recruited and immediately fused to the plasma membrane by stimulation; *Resting newcomer* (green), granules that are newly recruited, docked, and fused to the plasma membrane by stimulation. See also Shibasaki et al. (9) for details. *B*: Distribution of three modes of fusion events induced by 100 nmol/L glimepiride alone (open columns) and the combination of 100 nmol/L glimepiride and 10 nmol/L GLP-1 (closed columns). Data are expressed as means ± SEM of four to five independent experiments (n = 1–4 cells for each experiment). *$P < 0.05$ (Student unpaired t test).

to mediate the potentiation of insulin secretion through activation of phospholipase C-ε. Phospholipase C-ε activated by Rap1 possibly potentiates insulin secretion by promoting Ca^{2+}-induced Ca^{2+} release through the production of inositol triphosphate (32,33). Costimulation by sulfonylurea and cAMP has been found to induce a larger change in intracellular Ca^{2+} level than stimulation by sulfonylurea alone (34–36). On the contrary, a recent study (37) has shown that intracellular Ca^{2+} level is not a factor in the potentiation of tolbutamide-induced insulin secretion by an Epac-selective cAMP analog, 8-pCPT, in mouse pancreatic islets. However, our study of *Epac2A*$^{-/-}$ mice indicates that the intracellular Ca^{2+} is involved in the potentiation of insulin secretion by the combination of GLP-1 and glibenclamide (Fig. 6). The reason for the discrepancy between the recent study (37) and our present study is not clear at present, but might be due to the differences in the experimental conditions used. Since GLP-1 was reported to facilitate the inhibitory action of sulfonylurea at the K_{ATP} channels (34), Epac2A/Rap1 signaling may also contribute to promoting Ca^{2+} influx. Interactions of the K_{ATP} channel, Epac2A, Rim2α, and VDCC (38) also support this notion. It is possible that mechanisms other than the regulation of intracellular Ca^{2+} are also involved. We found by TIRFM analysis that costimulation by GLP-1 and sulfonylurea increased the number of fusion events derived from *resting newcomer* as well as *restless newcomer*, indicating that cAMP and sulfonylurea cooperatively promote the fusion of granules docked to the plasma membrane in addition to promoting the recruitment of granules from the cell interior to the plasma membrane. We have shown that the interaction of Epac2A and Rim2α is required for the potentiation by Epac-selective cAMP analog of glucose-induced insulin secretion (39). Rim2α is involved in the priming and docking to the plasma membrane of the insulin granule through interaction with Munc13–1 and Rab3A. The enhanced activation of Epac2A/Rap1 signaling may thus affect the docking and priming states of the insulin granules through interaction with Rim2α, which leads to an increase in the number of fusion events derived from *resting newcomer* and *old face*.

Epac2A has been found to be required for insulin secretion in response to the GLP-1 receptor agonist

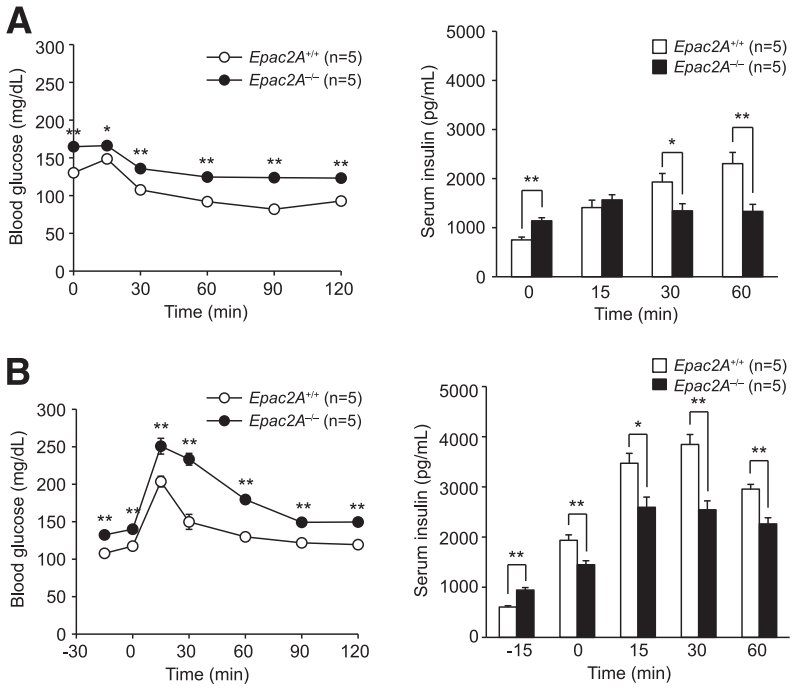

Figure 8—Insulin secretory response to coadministration of liraglutide and glimepiride is reduced in *Epac2A*$^{-/-}$ mice with diet-induced obesity. *A*: Effects of concomitant administration of liraglutide and glimepiride on blood glucose levels (left) and serum insulin levels (right) in *Epac2A*$^{+/+}$ mice (left, open circles; right, open columns) and *Epac2A*$^{-/-}$ mice (left, closed circles; right closed columns) fed an HFD. Liraglutide (300 μg/kg) and glimepiride (1 mg/kg) were administered at 0 min. *B*: Changes in blood glucose levels (left) and serum insulin levels (right) after oral glucose load following the concomitant administration of liraglutide and glimepiride in *Epac2A*$^{+/+}$ mice (left, open circles; right, open columns) and *Epac2A*$^{-/-}$ mice (left, closed circles; right, closed columns) fed an HFD. Liraglutide (300 μg/kg) and glimepiride (1 mg/kg) were administered at −15 min, and glucose (1.5 g/kg) was administered at 0 min. Data are expressed as means ± SEM (n = 5 for each group). *P < 0.05, **P < 0.01 (Student unpaired t test).

exendin-4 in vivo, indicating that Epac2A signaling is important for the blood glucose–lowering effects of incretin-related drugs (28). It has been reported (17,18) that combination therapies of DPP-4 inhibitors and sulfonylureas are often used for glycemic control in type 2 diabetes, but cause hypoglycemia in some cases. The incidence rate of hypoglycemia for DPP-4 inhibitors combined with gliclazide is lower than that for combination with glibenclamide or glimepiride (19). Our findings show that Epac2A/Rap1 signaling participates in the hypersecretion of insulin observed with combination therapies and suggest a mechanism for the sulfonylurea-dependent difference in the incidence rate of hypoglycemia.

In conclusion, we demonstrate the critical role of Epac2A/Rap1 signaling in the augmenting effect of incretins and sulfonylureas in insulin secretion, and we also find that such augmentation depends on the structures of the sulfonylureas. Our findings thus provide the basis for the effects of the combination therapies of incretin-related drugs and sulfonylureas in the treatment of type 2 diabetes.

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Author Contributions. H.T. designed the study, researched the data, and wrote, reviewed, and edited the manuscript. T.S. researched the data, contributed to the discussion, and wrote, reviewed, and edited the manuscript. J.-H.P. and D.-K.S. performed the high-fat–diet study and contributed to the discussion. S.H. and A.O. researched the data. T.T. researched the data and contributed to the discussion. S.S. contributed to the study design and the discussion and wrote, reviewed, and edited the manuscript. S.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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