Glucagon Promotes cAMP-response Element-binding Protein Phosphorylation via Activation of ERK1/2 in MIN6 Cell Line and Isolated Islets of Langerhans*

Stéphane Dalle‡, Christine Longuet‡, Safia Costes‡, Christophe Broca‡, Omar Faruque‡, Ghislaine Fontès‡, El Habib Hanif‡, and Dominique Bataille‡

From the ‡Unité INSERM U376, CHU Arnaud-de-Villeneuve, 34295 Montpellier Cedex 5 and §UMR 5160 CNRS, Institut de Biologie-Faculté de Médecine, 34060 Montpellier Cedex 1, France

Received for publication, November 14, 2003, and in revised form, February 18, 2004
Published, JBC Papers in Press, February 26, 2004, DOI 10.1074/jbc.M312483200

© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.

Glucagon, produced by post-translational processing of pre-glucagon in the pancreatic α cells, is one of the major metabolic hormones (1–4). Glucagon is released from α cells in the interrodinal state in a situation (hypoglycemia) that strongly differ from that in which incretins (3, 4), such as GIP or GLP-1, are released, that is during the early postprandial period preparing the β cell to the soon-coming glucose wave (5). Glucagon regulates the rate of glucose production in liver through both gluconeogenesis and glycogenolysis and consequently adjusts, in concert with insulin, blood glucose levels according to the needs of the organism (3, 4). To achieve its intracellular effects, glucagon binds to a glycoprotein receptor that spans the plasma membrane (1, 3, 6–8). The glucagon receptor (Gcgr) belongs to the class II (or B) family of heptahelical transmembrane G protein-coupled receptors (GPCRs) and is positively coupled to adenylate cyclase through the heterotrimeric G, protein (6–8). The hormone binds to the Gcgr on the cell surface, causing it to interact with stimulatory guanine nucleotide-binding regulatory protein G. This liberates the -subunit of G, to stimulate adenylate cyclase that catalyzes the conversion of ATP to cAMP, leading to activation of cAMP-dependent protein kinase A (PKA) (6–8).

Expression of functional Gcgrs in β cell lines, such as MIN6 (9) and INS-1 cells (10), and isolated rat pancreatic β cells (10) has been reported, and a role for glucagon in islet hormone secretion during feeding and fasting has been proposed (10, 11). Glucagon has been shown to potentiate the glucose-stimulated insulin release from β cell lines and from pancreatic islets (9, 11). In β cells models, glucagon increases cAMP, activates PKA which, in turn, phosphorylates the L-type voltage-dependent calcium channel (VDCC), leading to an increased glucose-induced calcium entry elicited through membrane depolarization, thus potentiating insulin secretion (9, 11). This observation is intriguing and paradoxical in that glucagon, which exhibits gluconeogenetic and glycogenolytic effects that are necessary for fighting against hypoglycemia, is also able to release insulin that drives the system in the opposite direction (3, 4, 9, 11). We have demonstrated recently (12) that miniglucagon, the C-terminal glucagon fragment also present in mature secretory granules of the α cells, is released together with glucagon in a low glucose situation (hypoglycemia). Miniglucagon completely blocks any possible insulinotropic action of glucagon, because this peptide is very potent in inhibiting insulin release by closing the β cell VDCC consecutively to plasma membrane repolarization (9, 12). Fully consistent with these findings, endogenously released glucagon from α cells has no effect on the magnitude of glucose-induced insuli

* This work was supported in part by the Association pour la Recherche sur le Cancer ARC (to S. D.), the Fondation pour la Recherche Médicale (to G. F.), the Université Montpellier-1, and the Conseil Régional du Languedoc-Roussillon. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Unité INSERM U376, CHU Arnaud-de-Villeneuve, 371, Rue du Doyen Gaston Giraud, 34295 Montpellier Cedex 5, France. Tel.: 33-4-67-41-52-30; Fax: 33-4-67-41-52-22; E-mail: bataille@montp.inserm.fr.

The abbreviations used are: Gcgr, glucagon receptor; CREB, cAMP-response element-binding protein; ERK1/2, extracellular signal-regulated kinase 1/2; PKA, cAMP-dependent protein kinase; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetate/acetoxymethyl ester; MAP, mitogen-activated protein; MEK, MAP kinase/ERK kinase; PI, phosphatidylinositol; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; GPCRs, G protein-coupled receptors; VDCC, voltage-dependent calcium channel.
lin secretion (13), although exogenous glucagon stimulates insulin secretion.

On the other hand, glucagon has been shown to exert important long-term effects on the pancreatic β cells. Glucagon contributes to the maintenance of the glucose-competent phenotype of native β cells, a key feature commonly impaired in physiopathology of type 2 diabetes (14), and is required for the differentiation of precursor cells into insulin-secreting β cells in the embryonic pancreas (15). Although attention has been focused on the biological role of glucagon in the pancreatic islet physiology, much less is known about the identity and the role of the downstream signaling pathways linked to the Gcgr present in the pancreatic β cells.

We first addressed the question of a possible positive coupling of endogenous β cell Gcgr, via cAMP-PKA pathway, to signaling components known to be linked to the tyrosine kinase receptor system. By using the MIN6 cell line and isolated rat pancreatic islets, we report that glucagon treatment of β cells caused a time-dependent activation of ERK1/2 at a low glucose concentration. We used pharmacological approaches to identify the signaling pathway leading from Gcgr activation to ERK1/2 activation. We observed that glucagon-induced MEK-ERK1/2 activities are mediated by the cAMP-PKA pathway but independently from p21ras, PI 3-kinase, and extracellular calcium influx. An increase in intracellular Ca2+ through mobilization of intracellular calcium pools was required for a maximal activation of ERK1/2 by PKA. In addition, Gcgr internalization process appears to participate in the ERK1/2 activation. Through nuclear translocation of ERK1/2, we found that glucagon induces phosphorylation of CREB, a major transcription factor allowing, by regulation of gene expression, the maintenance of glucose-competent phenotype of the β cell, and the disruption of which in the β cell leads to type II diabetes (16, 17). In a second part of our study, we evaluated whether or not miniglucagon, which we have shown to be co-released with glucagon from a cell (12), exerts a counter-regulation on the signaling network engaged by Gcgr in the β cells. Most interesting, we found that miniglucagon, which blocks the insulinotropic effect of glucagon (9, 12), does not inhibit the glucagon-induced PKA/ERK1/2/CREB signaling pathway leaving intact the glucagon access to the β cell transcription machinery.

EXPERIMENTAL PROCEDURES

Materials—Glucagon, somatostatin, and 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetate/acetoxymethyl ester (BAPTA/AM) were purchased from Calbiochem. Glucagon-like peptide-1 (7–36)-amide (GLP-1) was from Peninsula Laboratories (San Carlos, CA). Miniglucagon was synthesized in our laboratory (9). Mouse monoclonal anti-phospho-ERK1/2 (p44/42 MAP kinase) antibody, which selectively recognizes the doubly phosphorylated, active forms of these kinases, mouse monoclonal anti-phosphoserine 133-CREB antibody, which detects endogenous levels of CREB specifically when phosphorylated at Ser133 (and also the phosphorylated form of the CREB-related protein ATF-1), and rabbit polyclonal anti-CREB antibody were obtained from Transduction Laboratories (Lexington, KY). Horseradish peroxidase-linked anti-rabbit and anti-mouse antibodies, antiphosphotyrosine (PY20) antibody, and protein A/G-Plus-agarose were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Dulbecco’s modified Eagle’s medium and fetal calf serum were purchased from Invitrogen. Nitrocellulose transfer membranes (Protran) were purchased from Schleicher & Schuell. 45CaCl2 was obtained from Amersham Biosciences. All other reagents were purchased from Sigma.

Pancreatic Islet Preparation and Cell Culture—Pancreatic islets were isolated from fed male Wistar rats (Iffa-Credo, France) weighing 280–320 g at the day of sacrifice. Islets were isolated by collagenase digestion followed by Ficoll gradient separation, as described previously (18). For Western blotting experiments, pancreatic islets were stabilized for 2 h at 37 °C in HEPES-balanced Krebs-Ringer bicarbonate buffer (119 mM NaCl, 4 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2.5 mM CaCl2, 20 mM HEPES, pH 7.2) containing 0.1% bovine serum albumin (BSA) (KRB buffer) and 2.8 mM glucose, either in the absence or the presence of inhibitor. Islets were then washed and further incubated by groups of 100 islets for 10 min at 37 °C in KRB buffer supplemented with stimulants and inhibitor as described in the figure legends. Following the 10-min incubation, islets were rapidly centrifuged, and 1 μl islet of cold lysis buffer (50 mM HEPES, 1% Nonidet P-40, 1 mM Na3VO4, 100 mM NaF, 10 mM glyrophosphate, 4 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml aprotinin) was added. After 30 min of incubation in lysis buffer, islets were sonicated (10 s) and stored at –20 °C until use for subsequent protein determination by Bradford assay and Western blotting experiments. MIN6 cells were cultured as described previously (9, 16, 19) in Dulbecco’s modified Eagle’s medium containing 25 mM glucose supplemented with 15% fetal calf serum, 100 μg/ml streptomycin, 100 units/ml penicillin sulfate, and 50 μg/ml β-mercaptoethanol. Cultures were never allowed to become confluent. Each batch of MIN6 cells used in the experiments was tested for the dose-dependent insulin response to glucagon that was always in the physiological range (9).

Measurement of CAMP Production—MIN6 cells were grown in 24-well plates and incubated in Dulbecco’s modified Eagle’s medium without fetal calf serum containing 1% BSA, 1 mM isobutylmethylxanthine as an inhibitor of cAMP phosphodiesterase, and the test substances. After a 10-min incubation at room temperature, cells were extracted using perchloric acid. A sample was neutralized and succinylated to increase the sensitivity of the assay, and cAMP was quantified by radioimmunoassay (9).

Measurement of Ca2+ Influx—MIN6 cells were preincubated for 30 min at 37 °C in KRB containing 0.1% BSA and 1 mM glucose in a 5% CO2 environment. The preincubation solution was then replaced by KRB containing 8 μM 45CaCl2 (5–50 mM CaCl2) and the test agents. The reaction, performed at 37 °C, was stopped by aspiration of the medium. Cells were then washed with ice-cold buffer (135 mM NaCl, 5 mM KC1, 2.5 mM CaCl2, 10 mM Hepes) and solubilized in KRB containing 0.1% Triton X-100 for 1 h at room temperature. An aliquot of the solution (100 μl) was then assayed for 45Ca2+ content in a scintillation medium (Complete Phase Combining system, Amersham Biosciences).

Western Blotting and Immunoprecipitation—After a 2-h stabilization period at 37 °C in KRB buffer (16, 19), either in the absence or the presence of inhibitors, 6-well plates of MIN6 cells (60–80% confluency) were incubated in KRB containing different glucose concentrations and other test agents for various times as indicated in figure legends. MIN6 cells were then lysed in a cold lysis buffer containing 50 mM HEPES, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1 mM Na3VO4, 1 mM PMSF, 10 mM glyrophosphate, 100 mM NaF, and 1 mg/ml bacitracin. After a 30-min incubation in lysis buffer, cell lysates were centrifuged at 14,000 rpm for 30 min to remove insoluble materials. Protein content was determined by Bradford assay. Cell lysates were denatured by boiling for 3 min in Laemmli sample buffer containing 100 mM dithiothreitol. Equal amounts of lysate proteins (25–35 μg of protein per lane) were resolved by SDS-PAGE. For immunoprecipitation, the supernatants (400–800 μg of total protein) were incubated with primary antibody as indicated for 4 h at 4 °C. Immunocomplexes were precipitated from the supernatant with protein A/Plus agarose, washed three times with ice-cold cell lysis buffer, and boiled for 3 min in Laemmli sample buffer and resolved by SDS-PAGE. Nitrocellulose membranes were blocked, probed with the specific antibodies, and incubated with horseradish peroxidase-linked secondary antibody followed by enhanced chemiluminescence detection. Visualization and quantification of the bands were performed using a Kodak Image Station 2000 system (Eastman Kodak Co.).

Immunostaining for Phospho-p44/42 MAP Kinase—MIN6 cells were grown on glass coverslips for 3–5 days. Cells were fixed with 3.7% formaldehyde for 30 min at 4 °C. Cells were permeabilized by incubation with 0.1% Triton-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100 (normal horse serum in TBST buffer) for 1 h at room temperature, coverslips were then washed followed by the anti-phospho-ERK1/2 primary antibody in 5% BSA TBST buffer overnight at 4 °C at 1:400 dilution. After washing, phospho-ERK1/2 antibody was detected by incubation with fluorescein isothiocyanate-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Burlingame, CA) diluted 1:100 in 3% BSA TBST buffer, for 1 h at room temperature. Coverslips were mounted using a polyvinyl alcohol medium at least 1 h before observation using a Zeiss dual photon confocal microscope (Oberkocher, Germany).

Subcellular Fractionation—After 2 h of starvation in KRB, 10-cm
MIN6 cells were stimulated with 10 nM glucagon for 5 min. Cells are washed twice with ice-cold phosphate-buffered saline and scraped in 500 μl of hypotonic buffer (10 mM HEPES; 10 mM NaCl; 1 mM KH₂PO₄; 5 mM NaHCO₃; 1 mM CaCl₂; 0.5 mM MgCl₂; 5 mM EDTA, pH 8; 1 mM PMSP; 10 μg/ml aprotinin; 10 μg/ml leupeptin; 1 μg/ml pepstatin). After 15 min of incubation, cells were disrupted (50 times) using a Dounce homogenizer (pestle B) at 4°C and centrifuged for 5 min at 7500 rpm at 4°C. Supernatant containing cytosol and membranes was collected and preserved for protein content determination and Western blot analysis, and the pellet containing nuclei was disrupted (30 times) using a Dounce homogenizer (pestle B) in 10 mM Tris, pH 7.5, 300 mM sucrose, 1 mM EDTA, pH 8, 0.1% Nonidet P-40, 1 mM PMSP, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 μg/ml pepstatin (TSE buffer) and centrifuged for 5 min at 5000 rpm at 4°C. Final pellet containing pure nuclei was dissolved in TSE buffer for protein determination before denaturation in Laemmli buffer and analysis by SDS-PAGE.

**RESULTS**

**ERK1/2 Phosphorylation by Glucagon in MIN6 Cells**—We have demonstrated previously (9) that activated Gcgrs induce cAMP production through adenylate cyclase activation in MIN6 cells. Because the cAMP/PKA pathway has been shown to activate the ERK1/2 signaling cascade in β cells (19, 20, 21), we evaluated whether glucagon activates ERK1/2 under non-insulinotropic (low) glucose concentration (2.8 mM), which corresponds to physiological conditions for glucagon release from pancreatic β cells in vivo, when glucagon is present in extracellular spaces inside the islet at the vicinity of the insulin-secreting β cells (3, 4). In this glucose situation, glucagon has no effect on insulin secretion (9, 12, 13). As shown in Fig. 1 (A and B), in the presence of a non-insulinotropic glucose concentration (2.8 mM), MIN6 cells exhibited a small but significant transient phosphorylation of ERK1/2 (Fig. 1A). The amounts of phosphorylated ERK2 (p42 MAP kinase) peaked at 5 min and returned to the basal level by 10–20 min (Fig. 1B). Addition of 10 nM glucagon significantly enhanced glucose-induced activation of ERK1/2 at all time points (Fig. 1, A and B). We also tested the hypothesis whether activated Gcgrs transduce ERK1/2 cascade in MIN6 cells in the absence of glucose. Most interesting, we observed that, even under these extra-physiological conditions, glucagon alone induced a rapid and transient phosphorylation of ERK1/2 with a maximal effect observed at 5 min which returned to basal by 30 min (Fig. 1, C and D).

**Mechanisms of Glucagon-induced ERK1/2 Activation**—We used pharmacological approaches to identify the mechanisms of glucagon-induced ERK1/2 activation in the absence of glucose during the glucagon stimulation. We tested the hypothesis that, under non-physiological conditions in which there is no calcium influx, a mechanism linked to the cAMP/PKA pathway exists in the β cells, activating the ERK1/2 cascade independ-
ently from calcium entry. In line with the fact that glucagon alone had no significant effect on basal calcium uptake (Fig. 7A), we observed that glucagon-mediated ERK1/2 activation in the absence of glucose (10 min) (Fig. 2, A and B), or in the presence of 2.8 mM glucose (data not shown), was not significantly inhibited by the VDCC blocker nifedipine. The role of PKA in the ERK1/2 activation by glucagon was addressed in this context. The complete suppression of the glucagon-stimulated activation of ERK1/2 requires PKA activation (Fig. 2, A and B). This observation excludes the possibility that activation of ERK1/2 signaling cascade by Gcgr in β cells is mediated through cAMP independently from PKA. As a control, H89 pretreatment had no inhibitory effect on epidermal growth factor-induced ERK1/2 phosphorylation (data not shown). For comparison, we tested the H89 treatment on GLP-1-induced ERK1/2 activation. As shown in Fig. 2A, we found that the H89 treatment inhibited only 60–70% of GLP-1-induced ERK1/2 activation, suggesting that others pathways,
independent from PKA, participate in the activation of the ERK1/2 cascade by GLP-1 in β cells, consistent with the work of Arnette et al. (21). We also evaluated the role of MEK (the upstream kinase of ERK1/2) by using a specific inhibitor, PD98059. As shown in Fig. 2 (C and D), a treatment with PD98059 completely suppressed glucagon-dependent ERK1/2 activation, indicating that the upstream MEK activity is necessary for the activation of ERK1/2 by glucagon. As PKA is capable of regulating intracellular [Ca²⁺] through the release of intracellular pools, we used an intracellular calcium chelator, BAPTA/AM, to test the hypothesis that a reduction in intracellular [Ca²⁺] affected ERK1/2 activation by glucagon. As shown in Fig. 2 (E and F), glucagon-stimulated ERK1/2 phosphorylation was partially blocked when cells were pretreated with BAPTA/AM. As a whole, these results suggest that intracellular [Ca²⁺] does not appear to be essential for glucagon-induced ERK1/2 phosphorylation but is required for an optimal activation.

We also investigated the role of PI 3-kinase, which displays some upstream input into ERK1/2 in several cellular systems (22). As shown in Fig. 2 (G and H), activation of ERK1/2 by glucagon remained unaffected after a wortmannin pretreat-
ment, indicating that PI 3-kinase is not involved in the glucagon-induced ERK1/2 activation. As a positive control, wortmannin pretreatment abolished the protein kinase B/Akt phosphorylation induced by insulin (data not shown).

Finally, we investigated the involvement of p21 Ras in the activation of the ERK1/2 signaling cascade by glucagon. Activation of p21 Ras is known to be dependent on Ras-specific guanine nucleotide exchange factors that are activated by the phosphorylated active form of Shc proteins or by cAMP independently from PKA (23–25). First, we have shown that glucagon-stimulated ERK1/2 activation was mediated via cAMP-activated PKA (Fig. 2, A and B) and, therefore, not through other cAMP downstream effectors. Second, we investigated whether glucagon induced phosphorylation of Shc proteins. By using an immunoprecipitation approach, we found that glucagon failed to induce tyrosine phosphorylation of Shc proteins (Fig. 2I). As a control, epidermal growth factor significantly induced tyrosine phosphorylation of Shc (52- and 46-kDa isoforms). Taken together, these data exclude the involvement of p21 Ras in glucagon-induced ERK1/2 activation in H9252 cells.

**GR Does Not Switch Its G Protein Coupling Specificity from Gs to Gi, but Its Internalization Process Is Involved in ERK1/2 Activation**

It has been shown that the β2-adrenergic receptor switches its G protein coupling specificity from Gs to Gi upon PKA-dependent phosphorylation leading to activation of ERK1/2 (26). Pertussis toxin, an irreversible Gi/Go-specific antagonist that uncouples receptor from G protein, was used to determine whether Gcgr couples to pertussis toxin-sensitive G proteins in relation to ERK1/2 activation. Preincubation with pertussis toxin did not inhibit ERK1/2 phosphorylation by glu-

---

**FIG. 3. Gcgr internalization process is involved in ERK1/2 signaling activation by glucagon.** Western blot analyses of phospho-ERK1/2 and total ERK in MIN6 cells following incubation for 10 min in absence or in presence of glucagon, or lysophosphatidic acid (LPA), without or with inhibitor (A and B). Gαo/Gαs-specific antagonist pertussis toxin (100 ng/ml) was added overnight and maintained during the stabilization and agonist stimulation period. Clathrin-mediated receptor endocytosis inhibitor, dansylcadaverine (Dsc) (50 μM) was added 30 min before glucagon stimulation (C and D). 25 μg of cell lysates was subjected to 10% SDS-PAGE and immunoblotted with the anti-phosphorylated-ERK1/2 antibody. Typical autoradiograph representative of five experiments is shown. *, p < 0.05; **, p < 0.01.
Glucagon Signals to β Cell Nucleus

Glucagon induces the nuclear translocation of ERK1/2 in MIN6 cells. A and B, 2-h KRB-stabilized MIN6 cells were incubated with KRB alone (control) or with 10 nM glucagon or 10 mM glucose (stimulated) for 10 min. Nuclear (N) and cytosol (C) fractions were prepared as described under “Experimental Procedures.” Equal amounts of protein were loaded in each lane. Immunoblots with active-phosphorylated form of ERK1/2 (A) and total ERK1/2 (B). For immunofluorescence studies of active ERK1/2 in MIN6 cells, following no glucagon stimulation (basal) (C) or a 10-min glucagon (10 nM) stimulation period (D), MIN6 cells were fixed and permeabilized, and active phospho-ERK1/2 immunostaining was performed as described under “Experimental Procedures.”

FIG. 4. Glucagon induces the nuclear translocation of ERK1/2 in MIN6 cells. A and B, 2-h KRB-stabilized MIN6 cells were incubated with KRB alone (control) or with 10 nM glucagon or 10 mM glucose (stimulated) for 10 min. Nuclear (N) and cytosol (C) fractions were prepared as described under “Experimental Procedures.” Equal amounts of protein were loaded in each lane. Immunoblots with active-phosphorylated form of ERK1/2 (A) and total ERK1/2 (B). For immunofluorescence studies of active ERK1/2 in MIN6 cells, following no glucagon stimulation (basal) (C) or a 10-min glucagon (10 nM) stimulation period (D), MIN6 cells were fixed and permeabilized, and active phospho-ERK1/2 immunostaining was performed as described under “Experimental Procedures.”

In β cells exposed to glucagon (Fig. 4C) compared with basal (Fig. 4D), ERK1/2 immunostaining was present mostly in the cytoplasm and under the form of strong spots in the nucleus. These data are consistent with the results of subcellular fractionation. Thus, Gcgr engages signal transduction networks in the β cells that convey extracellular signal from the cell surface to nucleus, most probably inducing a biological response at the genetic level.

Existence of the ERK1/2 Signaling Network Engaged by Glucagon in Islets of Langerhans—In order to confirm, using another model, the ERK1/2 signaling network engaged by glucagon evidenced in MIN6 cells, we studied this pathway in isolated islets of Langerhans. As shown in Fig. 5, in the presence of 2.8 mM glucose, glucagon induced activation of the ERK1/2 signaling cascade in rat isolated islets, and a PD98059 treatment eliminates the ERK1/2 phosphorylation elicited by glucagon, thus confirming the data obtained in the MIN6 cells. Therefore, these data, fully consistent with a previous report (10) showing the expression of functional Gcgrs in isolated rat islets, show that Gcgrs are positively coupled to the ERK1/2 signaling network in islets.

Activation of CREB by Glucagon—We next investigated whether, in a situation of non-insulinotropic glucose concentration (2.8 mM), the glucagon effect on ERK1/2 signaling cascade, exerted via cAMP/PKA independently from calcium entry, leads to activation of transcription factors. One of the best characterized stimulus-induced transcription factors, the cAMP-response element-binding protein (CREB), activates transcription of target genes in response to various stimuli, including peptide hormones that induce activation of a variety of protein kinases including PKA and ERK1/2 (33). These kinases induce phosphorylation of CREB at a particular residue, Ser133, and phosphorylation of Ser133 is required for CREB-mediated stimulation of transcription of many genes considered as essential for the glucose-responsive β cell phenotype (16, 17, 33). Therefore, we tested the hypothesis whether glucagon induces phosphorylation of this transcription factor, and we evaluated the involvement of the PKA/ERK1/2 signaling network in this process. As shown in Fig. 6A, we found that, in the presence of 2.8 mM glucose, glucagon stimulated the phosphorylation of CREB at Ser133 with a maximal effect observed at 10–20 min and a 30-min duration. The lack of any inhibitory
effect of nifedipine suggests that glucagon-induced CREB activation is independent from calcium influx (Fig. 6B), as shown for ERK1/2 activation (Fig. 2A); this is consistent with the fact that glucagon has no significant effect on basal calcium uptake (Fig. 7B). We also found that activation of CREB by glucagon was completely blocked by PD98059 showing that phosphorylation of CREB by glucagon in the \( \beta \) cells requires ERK1/2 activation by MEK (Fig. 6C). Most interesting, another class II GPCR, GLP-1 receptor (GLP-1R), when activated, induced CREB phosphorylation but independently from the ERK1/2 cascade, indicating differences between these two GPCRs, Gcgr and GLP-1R, in signaling pathway leading to CREB phosphorylation in \( \beta \) cells (Fig. 6C). As CREB phosphorylation induced by glucagon was maintained up to 30 min while ERK1/2 activation was transient (Fig. 1, A and B), we addressed the question of a possible involvement of an additional kinase, such as PKA, to maintain the glucagon-induced CREB phosphorylation. We tested the effect of PD98059 at time points beyond 10 min (30 min) and found that CREB phosphorylation induced by glucagon was eliminated by the MEK inhibitor treatment (data not shown).

**Role of Miniglucagon on Glucagon-induced ERK1/2 and CREB Activation**—We next determined whether the ERK1/2 and the subsequent CREB activation by glucagon are under the control of miniglucagon, which we have shown to be released together with glucagon from the \( \beta \) cells (12). We first evaluated the effect of miniglucagon on second messengers production induced by glucagon in \( \beta \) cells, such as extracellular calcium influx and cAMP, in the presence of a low glucose concentration. Glucagon has no effect on extracellular calcium uptake in this situation (Fig. 7A). On the other hand, glucagon induced the production of cAMP in \( \beta \) cells, which was not inhibited by the miniglucagon exposure (Fig. 7B). As a positive control, we verified that miniglucagon was efficient in inhibiting glucose-induced extracellular calcium influx (data not shown), as reported previously (9).

Second, we evaluated the effect of miniglucagon on glucagon-
induced ERK1/2 and CREB phosphorylation. As shown in Fig. 7C, miniglucagon failed to inhibit the glucagon-activated ERK1/2 signaling cascade, which has been shown to be strictly dependent on PKA activation and independent on calcium influx (Fig. 2, A and B). For comparison, we used somatostatin, another potent inhibitor of insulin secretion which acts by blocking calcium influxes via direct closure of VDCC and inhibition of cAMP production in the β cells (34). Unlike miniglucagon, somatostatin blocked the cAMP production induced by glucagon (Fig. 7D) and inhibited glucagon-induced ERK1/2 activation (Fig. 7E), which has been shown to be dependent on glucagon-induced cAMP production in this glucose situation. Finally, we found that the glucagon-induced Ser133 phosphorylation of CREB mediated by the ERK1/2 signaling cascade was not modified by miniglucagon, whereas it was inhibited by somatostatin (Fig. 7F).

**DISCUSSION**

The Gcgr, widely used as a model of Gs-coupled GPCR, activates adenylyl cyclase increasing intracellular cAMP and intracellular [Ca2+]. In this report, we show that, in a situation of low glucose concentration which corresponds in vivo to glucagon release from a cells, glucagon activation of cAMP-dependent PKA, in concert with an increase in intracellular [Ca2+] and independently from extracellular calcium influx, exerts a full stimulatory effect of MEK and ERK1/2 activation in β cells. The major finding of this paper is that the ERK1/2 signaling cascade was not modified by miniglucagon, whereas it was inhibited by somatostatin (Fig. 7F).
network engaged by the β cell Gcgr leads to phosphorylation of the transcription factor CREB, shown to be crucial for β cell survival and phenotype (16, 17), and that this cellular response is well coordinated to miniglucagon effects.

Intracellular cAMP is an important second messenger that allows Gcgr to activate downstream molecular targets, such as PKA, for protein phosphorylation and subsequent activation (1, 3, 9). Depending on the cell type, PKA regulates ERK1/2 activity by using various pathways. In the p21ras-dependent Raf-MEK-ERK1/2 signaling cascade, PKA inhibits Raf-1, suppressing ERK1/2 activation for cell proliferation (35). On the other hand, in PC12 cells, PKA shuts off Raf-1 but stimulates ERK1/2 through activation of B-Raf by the p21ras-related G protein Rap1 (36), PKA phosphorylates Rap1, and this was assumed to be the molecular mechanism for B-Raf activation and subsequent ERK1/2 stimulation (35, 36). Here we report that a PKA inhibitor completely inhibited glucagon-induced ERK1/2 activation. Hence, in β cells, glucagon-stimulated ERK1/2 activation is mediated by cAMP-activated PKA pathway but was independent of other cAMP molecular targets. As Rap-1 and B-Raf have been shown to be present in MIN6 cells (19, 37), it is possible that glucagon stimulates ERK1/2 by influencing the B-Raf activity through PKA-mediated phosphorylation of Rap-1, as shown for others GPCRs (19, 37).

In many cells, cAMP and intracellular [Ca^{2+}] seconds messengers are tightly coupled. Increased intracellular [Ca^{2+}] has been shown to stimulate the ERK1/2 signaling cascade (23, 24, 38). We found that glucagon induced a rise in intracellular [Ca^{2+}] in β cells which resulted from mobilization of intracellular pools of calcium (and not from extracellular calcium influx), most probably via PKA activation as shown in other cell types (39), and participates in activation of ERK1/2. In this low glucose situation, it is likely that glucagon-activated PKA phosphorylates the VDCCs that remain in a closed position in the absence of glucose-induced membrane depolarization. Consistent with this hypothesis, we found that, in a situation of low, non-insulinotropic glucose concentration, glucagon failed to induce calcium influx while PKA is active. Hence, glucagon activates the ERK1/2 signaling cascade via cAMP-dependent PKA and an increase in intracellular [Ca^{2+}] resulting from the release of intracellular calcium pools. Because a rise in intracellular [Ca^{2+}] has been shown to activate Raf-1 (23, 24, 38), a glucagon-mediated increase in intracellular [Ca^{2+}] may alternatively play a synergistic role with PKA in ERK1/2 activation by preventing PKA from inhibiting Raf-1, as shown in other cell types (35).

Gcgr is a member of class II GPCRs that shares little amino acid sequence homology with the larger class I GPCR family (6, 8). However, as reported previously (28, 29) for the class I β-adrenergic receptor, the clathrin-mediated endocytosis process of Gcgr may participate in the activation of ERK1/2 signaling cascade. Thus, another major findings of this paper is that the Gcgr internalization process in the β cells appears as a signaling event in which the receptor may function as a structural component of the ERK1/2 signaling complex, and that the two structural classes of GPCRs, I and II, are not functionally distinct. Internalization, homologous desensitization, and participation of cytoplasmic scaffold proteins linking the β cell Gcgr to signaling intermediates and intracellular effectors, such as ERK1/2, remain to be elucidated.

According to subcellular fractionation and immunofluorescence data, it clearly appears that glucagon induces a significant portion of the amount and the activity of ERK1/2 to translocate to the nucleus. However, we found that the majority of phosphorylated-activated ERK1/2 remains in the cytosolic fraction. Because CREB has been shown to be almost exclusively located in the nucleus (33, 40–42), glucagon-induced CREB phosphorylation, mediated by activated ERK1/2, most probably takes place in the nucleus. One substrate of ERK1/2 is the ribosomal protein S6 kinase of 90 kDa (p90Rsk) that has been reported to phosphorylate CREB at Ser-133 (33, 43, 44). On activation, it is highly possible that both ERK1/2 and p90Rsk translocate to the nucleus where activation of p90Rsk by ERK1/2 leads to phosphorylation of CREB. However, we cannot exclude the possibility that p90Rsk is activated by the cytosolic fraction of phosphorylated ERK1/2 and translocates to the nucleus to mediate CREB phosphorylation.

It must be noted that, in the presence of a non-insulinotropic glucose concentration (interprandial states), glucagon is released under hypoglycemic conditions from the surrounding α cells which, for a large part, are in direct contact with the β cells inside the islet (1). In this glucose situation, glucagon does not stimulate insulin release (9, 12, 13). However, we report that glucagon activates the ERK1/2 pathway in β cells and regulates, through serine-phosphorylation, the transcription factor CREB which is crucial for β cell differentiation and survival, the disruption of which leads to metabolic abnormalities and type II diabetes (16). Thus, based on our results, this new regulatory mechanism in β cell engaged by glucagon is now suspected to participate in the control of the β cell physiology, in particular the maintenance of the glucose-compotent phenotype by inducing expression of a number of early response genes known to be under the control of CREB and implicated in β cell survival and differentiation (16, 17). Glucagon-induced CREB function in β cells might also exert a permissive effect on incretin peptides or growth factor through induction of gene expression. However, because the observation that CREB is phosphorylated implies but does not necessarily mean that regulation of transcriptional machinery, analysis of the set of genes under the control of this signaling network recruited by glucagon, deserves further investigation. One of these has been clearly identified recently by Jhala et al. (16) and seems to be β cell IRS2.

Moreover, co-secreted miniglucagon from α cells prevents, by maintaining the VDCCs in a closed position (9, 12), any possible concomitant stimulatory effect of glucagon on insulin secretion that would deeply impair the peripheral effect of glucagon. On the other hand, of major importance is the fact that miniglucagon leaves completely untouched the cAMP production induced by glucagon and the subsequent beneficial PKA/ERK1/2 CREB signaling framework engaged in pancreatic β cell.

In summary, our data provide new insights in the regulation of pancreatic β cells by endogenous glucagon in concert with co-secreted miniglucagon. The following are now very important: 1) to dissect in more detail the molecular nature and functions of the kinases pathway/Gcgr complexes responsible for a downstream coordinated regulation of the ERK1/2 network in the β cells; 2) to identify the macromolecular multiprotein scaffolds underlying the cross-talks evidenced between Gcgr and tyrosine kinase receptor signals inside the β cell which, by their spatio-temporal specificities, represent “hot spots” which, if impaired, might be responsible for disorders in the β cell phenotype and biology, leading to type 2 diabetes.

REFERENCES
1. Unger, R. H., and Orci, L. (1997) in Ellenberg Rifkin’s Diabetes Mellitus, ed M. Eisenberg, W. B. Saunders Co., Philadelphia
2. Mojsov, S., Heinrich, G., Wilson, J. B., Ravazola, M., Orci, L., and Habener, J. F. (1980). J. Biol. Chem. 255, 11880–11889
3. Unger, R. H., and Orci, L. (1995) Endocrinology (de Groot, L. J., ed) 5th Ed., pp. 119–139, Elsevier Science Publishing Co., Inc., New York
4. Cryer, P. E. (1996) Horm. Res. 46, 192–194
5. Vlahos, T., Krapus, T., Madshid, S., and Holst, J. J. (2003) Regul. Pept. 114, 115–121
6. Jelinek, L. J., Lok, S., Rosenberg, G. B., Smith, R. A., Grant, F. J., Biggs, S.,...
Glucagon Signals to β Cell Nucleus

20355

Bensch, P., Kuijper, J. L., Sheppard, P. O., Sprecher, C. A., O'Hara, P. J., Foster, D., Walker, K. M., Chen, L. H. J., McKernan, P. A., and Kindsvogel, W. (1993) Science 259, 1614–1616

7. Birnbaumer, L., and Birnbaumer, M. (1994) J. Recept. Signal. Transduct. Res. 15, 213–252

8. Cypess, A. M., Unson, C. G., Wu, C. R., and Sakmar, T. P. (1999) J. Biol. Chem. 274, 19455–19464

9. Dalle, S., Smith, P., Blache, P., Le-Nguyen, D., Le Brigand, L., Bergeron, F., Ashcroft, F. M., and Bataille, D. (1999) J. Biol. Chem. 274, 10869–10876

10. Kieffer, T. J., Heller, R. S., Unson, C. G., Weir, G. C., and Habener, J. F. (1996) Endocrinology 137, 5119–5125

11. Kawai, K., Yokota, C., Ohashi, S., Watanabe, Y., and Yamashita, K. (1995) J. Biol. Chem. 270, 15507–15513

12. Dalle, S., Fontes, G., Lajoix, A. D., LeBrigand, L., Gross, R., Ribe, G., Dufour, M., Barry, L., LeNguyen, D., and Bataille, D. (2002) Diabetes 51, 406–412

13. Moens, K., Berger, V., Ahn, J. M., Van Schravendijk, C., Hruby, V. J., Pipeleers, D., and Schuit, F. (2002) Diabetes 51, 669–675

14. Haypen, P., Ling, Z., Pipeleers, D., and Schuit, F. (2000) Diabetologia 43, 1012–1019

15. Prasadan, K., Daune, E., Preuet, B., Spilde, T., Bhatia, A., Koeswadi, H., Hembree, M., Manna, P., and Gitten, G. K. (2002) Diabetes 51, 3220–3222

16. Jalha, U. S., Canettieri, G., Serafino, R. A., Kulkarni, R. N., Krajewski, S., Reed, J., Walker, J., Lin, X., White, M., and Montminy, M. (2003) Genes Dev. 17, 1575–1580

17. Habener, J. F., Vallejo, M., and Hoffer, J. P. (1989) Horm. Res. 32, 61–66

18. Lacy, P. E., and Kostianovskya, M. (1967) Diabetes 16, 35–39

19. Gomez, K., Pritchard, C., and Herbert, T. P. (2002) J. Biol. Chem. 277, 48146–48151

20. Fodor, M., Sekine, N., Roche, E., Filoux, C., Prentki, M., Wollheim, C. B., and Van Obberghen, E. (1995) J. Biol. Chem. 270, 7882–7889

21. Arnette, D., Gibson, T. B., Lawrence, M. C., January, B., Khoo, S., McGlynn, K., Vanderbilt, C. A., and Cobb, M. H. (2003) J. Biol. Chem. 278, 32517–32525

22. Chang, L., and Karin, M. (2001) Nature 410, 37–40

23. Kelch, W. (2000) Biochem. J. 351, 289–305

24. Morrison, D. K., and Cutler, R. E. (1997) Curr. Opin. Cell Biol. 9, 174–179

25. Busa, R., Abbe, P., Mantoux, F., Aberdam, E., Peyromaou, C., Eychene, A., Ortonne, J. P., and Balotti, R. (2006) EMBO J. 19, 2901–2919

26. Daaka, Y., Luttrell, L. M., and Lefkowitz, R. J. (1997) Nature 390, 88–91

27. Luttrell, L. M., Daaka, Y., Della Rocca, G. J., and Lefkowitz, R. J. (1997) J. Biol. Chem. 272, 31648–31656

28. Lefkowitz, R. J. (1998) J. Biol. Chem. 273, 18677–18680

29. Luttrell, L. M., Daaka, Y., and Lefkowitz, R. J. (1999) Curr. Opin. Cell Biol. 11, 177–183

30. Chow, J. C., Condorelli, G., and Smith, R. J. (1998) J. Biol. Chem. 273, 4672–4680

31. Benes, C., Raisin, M. P., van Tan, H., Creutz, C., Miyazaki, J., and Fagard, R. (1998) J. Biol. Chem. 273, 15507–15513

32. Khoo, S., and Cobb, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5599–5604

33. Shaywitz, A. J., and Greenberg, M. E. (1999) Annu. Rev. Biochem. 68, 821–861

34. Sharp, G. W. (1996) Annu. J. Physiol. 271, 1781–1799

35. Houslay, M. D., and Kolch, W. (2000) Mol. Pharmacol. 58, 659–668

36. Vossler, M. R., Yao, H., York, R. D., Pan, M. G., Rim, C. S., and Stork, P. J. (1997) Cell 89, 73–82

37. Ehses, J. A., Pelech, S. L., Pederson, R. A., and McIntosh, C. (2002) J. Biol. Chem. 277, 37088–37097

38. Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Plowman, G. D., Rudy, B., and Schlessinger, J. (1995) Nature 376, 737–745

39. Jiang, Y., Cypess, A. M., Chi, E. F., Wu, C. R., Unson, C. G., Merrifield, R. B., and Sakmar, T. P. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 10102–10107

40. Montminy, M. R. (1987) Nature 328, 175–178

41. Waeher, G., and Habener, J. F. (1991) Mol. Endocrinol. 5, 1431–1438

42. Ginty, D. D., Kornhauser, J. M., Thompson, M. A., Bading, H., Mayo, K. E., Tsakhaishi, J. S., and Greenberg, M. E. (1993) Science 260, 238–241

43. Xing, J., Ginty, D. D., and Greenberg, M. E. (1996) Science 273, 959–963

44. Chen, R., Sarnicki, C., and Blenis, J. (1992) Mol. Cell. Biol. 12, 915–927
