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GLP-1 Mediates Antiapoptotic Effect by Phosphorylating Bad through a β-Arrestin 1-mediated ERK1/2 Activation in Pancreatic β-Cells

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Strategies based on activating GLP-1 receptor (GLP-1R) are intensively developed for the treatment of type 2 diabetes. The exhaustive knowledge of the signaling pathways linked to activated GLP-1R within the β-cells is of major importance. In β-cells, GLP-1 activates the ERK1/2 cascade by diverse pathways dependent on either $G\alpha_s$/cAMP/cAMP-dependent protein kinase (PKA) or β-arrestin 1, a scaffold protein. Using pharmacological inhibitors, β-arrestin 1 small interfering RNA, and islets isolated from β-arrestin 1 knock-out mice, we demonstrate that GLP-1 stimulates ERK1/2 by two temporally distinct pathways. The PKA-dependent pathway mediates rapid and transient ERK1/2 phosphorylation that leads to nuclear translocation of the activated kinases. In contrast, the β-arrestin 1-dependent pathway produces a late ERK1/2 activity that is restricted to the β-cell cytoplasm. We further observe that GLP-1 phosphorylates the cytoplasmic proapoptotic protein Bad at Ser-112 but not at Ser-155. We find that the β-arrestin 1-dependent ERK1/2 activation engaged by GLP-1 mediates the Ser-112 phosphorylation of Bad, through p90RSK activation, allowing the association of Bad with the scaffold protein 14-3-3, leading to its inactivation. β-Arrestin 1 is further found to mediate the antiapoptotic effect of GLP-1 in β-cells through the ERK1/2-p90RSK-phosphorylation of Bad. This new regulatory mechanism engaged by activated GLP-1R involving a β-arrestin 1-dependent spatiotemporal regulation of the ERK1/2-p90RSK activity is now suspected to participate in the protection of β-cells against apoptosis. Such signaling mechanism may serve as a prototype to generate new therapeutic GLP-1R ligands.

GLP-1 (glucagon-like peptide-1), produced by post-translational processing of the proglucagon in enteroendocrine L-cells, is a potent gluco-regulatory peptide hormone. GLP-1 is released into the blood stream in response to nutrient ingestion, such as carbohydrates, amino acids, and fats, during the early postprandial period (1, 2). A major target for GLP-1 actions is the pancreatic β-cell. One of the main physiological roles of this endocrine hormone is to enhance insulin secretion in a glucose-dependent manner (1–5). Besides its insulinotropic action, GLP-1 also favors the maintenance of a correct β-cell glucose sensing, regulates transcriptional synthesis, induces β-cell proliferation, and is protective against apoptosis (6–9). Strategies based on activating GLP-1 receptor (GLP-1R)[3] are intensively developed for the treatment of type 2 diabetes and studies aiming at a better and exhaustive understanding of GLP-1 actions within the β-cells are of great importance (1–5).

GLP-1 exerts its intracellular effects through binding to its specific receptor that spans the plasma membrane. The GLP-1R belongs to the class II (or B) secretin/glucagon/vasoactive intestinal peptide superfamily of heptahelical transmembrane G protein-coupled receptors (GPCRs) (10, 11). The GLP-1R is positively coupled to adenylate cyclase, through $G\alpha_s$-containing heterotrimeric G-proteins, which catalyzes the conversion of ATP to cAMP, leading to the activation of second messenger pathways, such as cAMP-dependent protein kinase (PKA) and cAMP-regulated guanine nucleotide exchange factor (cAMP-GEF, also known as Epac) pathways (3, 10–16). Additionally, GLP-1 can activate a number of mitogenic kinases, such as phosphoinositide 3-kinase and p44/42 mitogen-activated protein kinases (also called ERK1/2) (7, 17–20).

The fine regulation of ERK1/2 activation is crucial for generating the appropriate physiological outcomes from a particular stimulus. Moreover, the duration and the intensity of the biological responses are in part controlled by the compartmentalization of ERK1/2 (21, 22). In β-cells, GLP-1 has been shown to activate the ERK1/2 cascade by diverse pathways dependent on influx of either calcium, $G\alpha_s$/cAMP/PKA, or β-arrestin 1, a scaffold protein (17–20, 23). However, up to now, how these pathways dictate the duration, the cytoplasmic *versus* nuclear location, and the ultimate functions of GLP-1-stimulated ERK1/2 activation remains totally unknown. Although many nuclear substrates have been shown to be phosphorylated by

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[3] The abbreviations used are: GLP-1R, glucagon-like peptide-1 receptor; GPCR, G protein-coupled receptor; PKA, cAMP-dependent protein kinase; ERK, extracellular signal-regulated kinase; p90RSK, p90 ribosomal S6 kinase; siRNA, small interfering RNA; DMEM, Dulbecco's modified Eagle's medium; KO, knock-out; WT, wild type; VDCC, voltage-dependent Ca^{2+} channel; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.

ERK1/2 in β-cells, little is known about the identity of ERK1/2 cytosolic substrates.

To address these issues, we examined the spatiotemporal regulation of ERK1/2 activation induced by GLP-1 and the potential requirement of ERK1/2 activity in phosphorylation of cytoplasmic targets. In this view, we studied Bad (Bcl-xL/Bcl-2-associated death promoter homolog), a well known ERK1/2 substrate in many cellular systems, localized in the β-cell cytoplasm (24–27). Bad (Bcl-xL/Bcl-2-associated death promoter homolog, or Bcl antagonist of cell death) was the first proapoptotic member of the Bcl-2 family to be described, and its proapoptotic activity is regulated by phosphorylation at several sites (24–28). Phosphorylation of Bad at Ser-112 favors binding of Bad to the scaffold protein 14-3-3 in the cytoplasm, preventing the translocation of Bad to mitochondria (28–30). This association further induces the release of Bcl-xL and Bcl-2 proteins from Bad, preventing accumulation of apoptotic proteins, such as Bax and Bak, on the mitochondrial membrane, blocking the release of cytochrome *c*, and inhibiting apoptosis (30, 31). In addition to its role in cell survival, Bad has been recently reported to play a pivotal role in the regulation of glucose-induced insulin secretion and β-cell mass (27). When phosphorylated at Ser-155, Bad nucleates a core complex at the mitochondrial membrane containing the glucokinase, a key component of the β-cell glucose-sensing machinery (27). Hence, it has been proposed that the phosphorylation state of Bad within the β-cells instructs Bad to assume a metabolic role by targeting glucokinase and regulating insulin secretion or alternatively a proapoptotic *versus* an antiapoptotic role (27). To date, the upstream signaling events that target Bad phosphorylation and thus control its functional behavior within the β-cells are unknown.

Here, we report that GLP-1 stimulates ERK1/2 by two temporally distinct pathways in β-cells. The rapid and transient first phase is mainly mediated by the $G\alpha_s$/cAMP/PKA pathway and favors the nuclear translocation of the ERK1/2. The sustained and long lasting second phase of ERK1/2 activation is exclusively β-arrestin 1 dependent, is restricted to the β-cell cytoplasm, and enhances the p90 ribosomal S6 kinase (p90RSK) activity. We further report that GLP-1 significantly phosphorylates Bad at Ser-112 but not at Ser-155. The β-arrestin 1-dependent ERK1/2-p90RSK signaling network engaged by GLP-1 phosphorylates Bad at Ser-112 and regulates its binding to the scaffold protein 14-3-3, revealing a new pathway in GLP-1-mediated antiapoptotic effects in β-cells.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Glucagon-like peptide-1-(7–36) amide (GLP-1-(7–36) amide) was obtained from Bachem (Bubendorf, Switzerland). Anti-CREB, anti-phospho-Bad (Ser-112), anti-phospho-Bad (Ser-155), anti-Bad, anti-tubulin, anti-p44/42 mitogen-activated protein kinase (ERK1/2), anti-phospho-p90RSK (Thr-573), anti-cleaved caspase-3, and anti-rabbit IgG horseradish peroxidase-linked antibodies were from Cell Signaling Technology (New England Biolabs, Beverly, MA). Anti-β-arrestin 1 and anti-ERK1 antibodies were from Transduction Laboratories (Lexington, KY). Horseradish peroxidase-linked anti-mouse, goat polyclonal anti-β-arrestin 1, anti-β-arrestin 2, anti-14-3-3 β (C-20) protein, anti-Bad (C7) antibodies, and protein A/G-Plus-agarose were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Lipofectamine^{TM}2000 and Stealth^{TM} prevalidated small interfering RNA (siRNA) double-stranded duplexes were from Invitrogen. Plasmids expressing hemagglutinin-tagged mouse wild type (WT) and mutant Bad S112A were a generous gift from Dr. Michael Greenberg (Harvard Medical School, Boston, MA) and Dr. Robert J. Lefkowitz (Duke University, Durham, NC). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were from Invitrogen. H89 and wortmannin were obtained from Calbiochem, and U0126 was from Promega (Madison, WI). $^{45}CaCl_2$ was obtained from Amersham Biosciences. All other reagents were purchased from Sigma.

Animals—β-Arrestin 1 knock-out (KO) mice, a generous gift from R. J. Lefkowitz, were generated on C57BL/6 background as described previously (32). WT littermate controls were C57BL/6 mice (5 weeks old) purchased from Charles River (France). All mice used in this study were treated in accordance with European Community guidelines, and the local institution approved the experimentation.

Pancreatic Islet Isolation, Western Blotting, and Insulin Secretion—Pancreatic islets were isolated from WT and β-arrestin 1 KO mice weighing 30–35 g on the day of killing using collagenase digestion followed by hand picking as described (33, 34). For Western blotting experiments, islets were stabilized for 2 h at 37 °C in HEPES-balanced Krebs-Ringer bicarbonate buffer containing 0.1% bovine serum albumin (KRB buffer) as described (33, 34) and supplemented with 1.4 mM glucose. Islets were washed and further incubated in groups of 100 islets for various times at 37 °C in KRB buffer supplemented with effectors. Islets were then rapidly centrifuged, washed with cold phosphate-buffered saline, frozen in N_2 liquid, and harvested in cold lysis buffer (1 μl/islet) (33, 34). After 30 min of incubation, islets were lysed by sonication (2 min) and stored at −20 °C until use. Samples were normalized for protein content by a Bradford protein assay. Equal amounts of lysate proteins were analyzed. For insulin secretion experiments, islets were preincubated for 30 min at 37 °C in KRB buffer supplemented with 1.4 mM glucose. Islets were further incubated in 1.4 or 16.7 mM glucose for 20 min. At the end of the stimulation, islets were pelleted by centrifugation and lysed in acid-ethanol for assessment of insulin content. Insulin secretion was quantified using an insulin radioimmunoassay kit purchased from Millipore (Billerica, MA).

Cell Culture and Insulin Secretion—MIN6 cells (passages 18–28) were cultured at 37 °C in 5% CO_2 in DMEM containing 25 mM glucose, 15% fetal calf serum, 100 μg/ml streptomycin, 100 units/ml penicillin sulfate, and 50 μM β-mercaptoethanol as described (35, 36). For insulin secretion experiments, MIN6 cells were plated in 24-well plates at a density of 1×10^6 cells/well for 3–5 days. The culture medium was changed 18 h before the experiments. Insulin secretion from MIN6 cells was performed in KRB buffer. The day of the experiment, cells were preincubated for 1 h in KRB buffer at 37 °C. MIN6 cells were then incubated for 2 h in KRB buffer containing various glucose concentrations with or without GLP-1. Insulin in supernatants was measured by a radioimmunoassay using ^{125}I-labeled insu-

lin, rat insulin standard, and anti-insulin porcine antiserum as described (35).

Determination of cAMP Production—MIN6 cells were grown in 24-well plates for 3–5 days under the same conditions as for insulin release. The medium was changed 1 day before the experiments. On the day of the experiment, cells were incubated in DMEM supplemented with 1% bovine serum albumin, 1 mM isobutylmethylxanthine as an inhibitor of cAMP phosphodiesterase and the test agents. After a 15-min incubation at room temperature, cells were extracted using 60% perchloric acid, the samples were neutralized with 9 N KOH succinylated to increase the sensitivity of the assay (37), and cAMP was quantified by a radioimmunoassay (35).

Measurement of Ca^{2+} Influx—24 h before the experiments, the culture medium was changed. The day of the experiment, cells were preincubated for 30 min at 37 °C in KRB buffer. The preincubation solution was then replaced by KRB buffer containing 8 μCi/ml $^{45}CaCl_2$ (5–50 mCi/mg calcium; Amersham Biosciences) and test agents. The reaction was stopped by aspiration of the medium. Cells were rapidly washed with ice-cold buffer (135 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM lanthanium chloride, 10 mM HEPES). The cells were then solubilized in KRB buffer containing 0.1% Triton X-100 for 1 h at room temperature. An aliquot of the solution was then assayed for $^{45}Ca^{2+}$ content in a β-counter after the addition of a liquid scintillation medium (Amersham Biosciences).

β-Arrestin 1 siRNA—β-Arrestin 1 expression was silenced in MIN6 cells using mouse 20–25-nt Stealth siRNA duplexes. The day before transfection, MIN6 cells were seeded in 12-well plates in regular growth medium without antibiotics and grown overnight to reach 30–40% confluence. The day of the experiment, Lipofectamine™2000-siRNA complexes were prepared according to the manufacturer's instructions. The final concentrations of siRNA tested in MIN6 cells were 25 (0.12 μg), 50 (0.24 μg), or 100 nM (0.48 μg) in each well. Cells were transfected with β-arrestin 1 siRNA or control siRNA (which corresponds to a non-targeting 20–25-nucleotide siRNA designed as a negative control) for 6 h before switching to fresh growth medium, including antibiotics. 72 h after transfection, cells were preincubated for 2 h at 37 °C in KRB buffer and then stimulated with pharmacological agents.

Western Blot—After a 2-h stabilization period at 37 °C in KRB buffer, 6-well plates of MIN6 cells (70% confluence) were incubated in KRB buffer containing GLP-1 and test agents for various times. MIN6 cells were then lysed in a cold lysis buffer (36). After a 30-min incubation, cell lysates were centrifuged at 13,000 rpm for 30 min. Supernatants were denatured by boiling for 5 min in Laemmli's sample buffer and resolved by SDS-PAGE. Samples were normalized for protein content by a Bradford protein assay, and equal amounts of lysate proteins were analyzed by Western blotting. Nitrocellulose membranes were blocked, probed with the specific antibodies, and incubated with horseradish peroxidase-linked secondary antibody followed by chemiluminescence detection.

Immunoprecipitation—After a 2-h stabilization period at 37 °C in KRB buffer, 10-cm dishes of MIN6 cells (70% confluence) were incubated in KRB buffer containing GLP-1 for various times as indicated. MIN6 cells were then lysed in a cold lysis buffer. Supernatants (250–800 μg of total proteins) were incubated with primary antibody for 4 h at 4 °C. Immunocomplexes were then precipitated from supernatant with protein A/G-Plus-agarose, washed three times with ice-cold cell lysis buffer, boiled for 5 min in Laemmli's sample buffer, and resolved by SDS-PAGE. Samples were normalized for protein content by a Bradford protein assay, and equal amounts of lysate proteins were analyzed by Western blotting.

Subcellular Fractionation—After a 2-h stabilization in KRB, 10-cm dishes seeded with MIN6 cells (70% confluence) were stimulated with GLP-1 for 5 or 20 min. Cells were then washed twice with ice-cold phosphate-buffered saline and scraped in 1.2 ml of hypotonic buffer (10 mM HEPES, 10 mM NaCl, 1 mM KH_2PO_4, 5 mM $NaHCO_3$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 1 mg/ml pepstatin). After a 15-min incubation, cells were Dounce homogenized 40 times on ice and centrifuged for 5 min at 1000 × *g* at 4 °C. Supernatants containing cytosol and membranes were collected and preserved for protein content determination and Western blotting. Pellets containing nuclei were Dounce homogenized 30 times in 10 mM Tris (pH 7.5), 300 mM sucrose, 1 mM EDTA (pH 8), 0.02% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 1 mg/ml pepstatin; centrifuged 5 min at 2500 × *g* at 4 °C; and rinsed twice. Pellets containing pure nuclei were then dissolved in Tris-buffered saline-sucrose/EDTA buffer for protein determination before Western blotting analysis.

Evaluation of Apoptosis in Pancreatic Islets—Apoptosis was evaluated by determination of histone-complexed DNA fragments (mono- and oligonucleosomes) with the cell death detection enzyme-linked immunosorbent assay kit (Roche Applied Science) according to the manufacturer's instructions.

Statistical Analysis—Results are expressed as mean ± S.E. for *n* independent experiments. Statistical differences between groups were evaluated by Student's *t* test for single comparisons or by analysis of variance, followed by the Newman-Keuls test in the case of multiple comparisons. Differences were considered significant at $p < 0.05$ (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

RESULTS

The Early Phase of ERK1/2 Activation Induced by GLP-1 in MIN6 Cells Is PKA-Dependent—In β-cells, insulinotropic glucose concentration has been shown to activate ERK1/2 by a rise in intracellular calcium concentration through the opening of voltage-dependent Ca^{2+} channels (VDCC) (18, 19, 33, 38). In order to avoid any interference of the nutrient effect on GLP-1 action, we tested the effects of GLP-1 alone on ERK1/2 activation in β-MIN6 cells. We found that GLP-1 induced a two-phase kinetic pattern of ERK1/2 activation, with a rapid and transient first phase (with maximal effect observed at 5 min) followed by a late second phase (10–60 min) remaining stable for up to 60 min (Fig. 1, *A* and *B*). Based on these results, we investigated the effect of GLP-1 at the 5 and 20 min time points in the subsequent experiments. Because GLP-1 is known to activate the ERK1/2 cascade by diverse signaling pathways, we

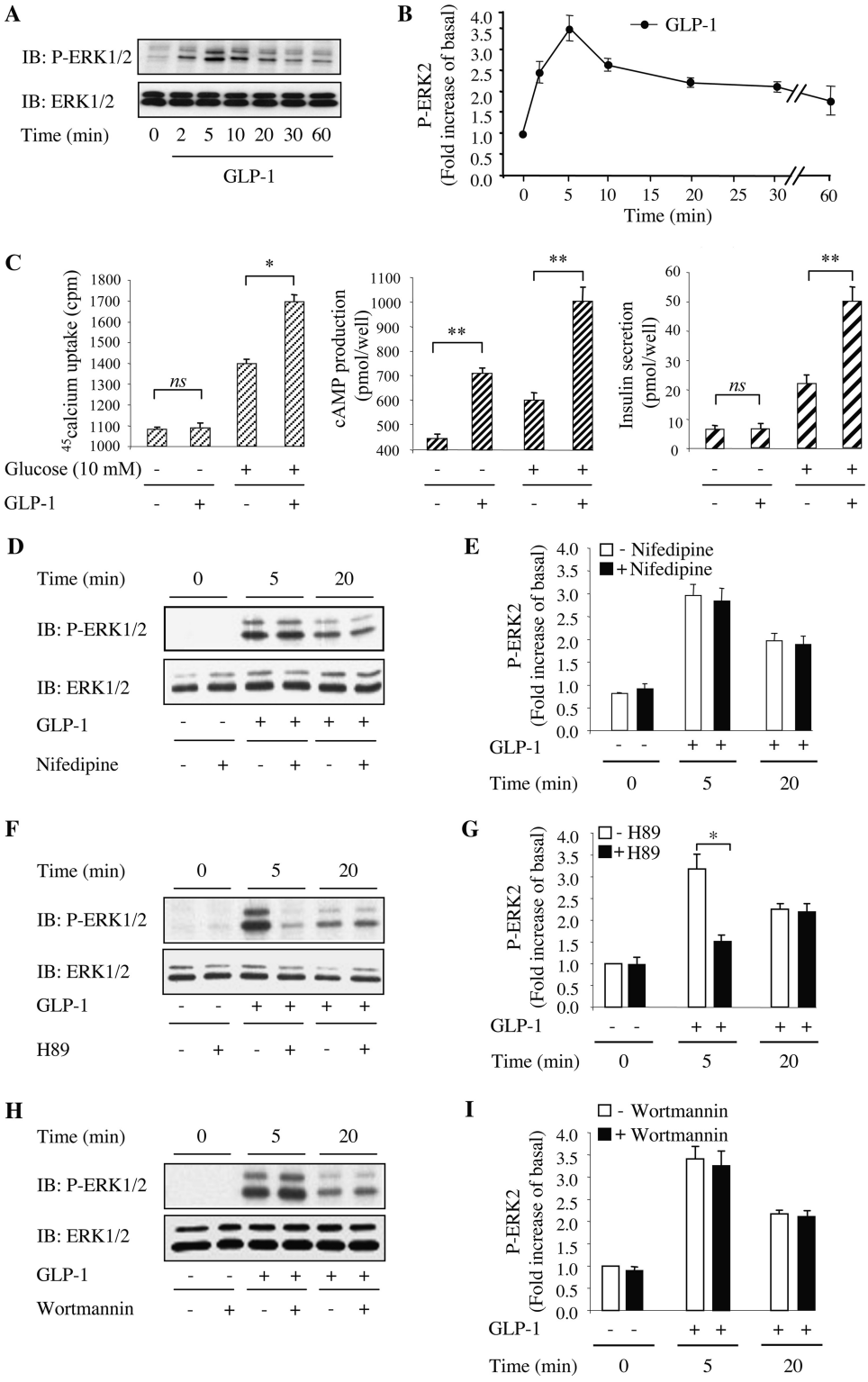

FIGURE 1. **Molecular mechanisms of GLP-1-induced ERK1/2 phosphorylation.** *A*, after a 2-h stabilization period in KRB buffer, MIN6 cells were treated with 10 nM GLP-1 for various times as indicated. Identical amounts of lysate proteins (25 μg) were subjected to 10% SDS-PAGE and immunoblotted (*IB*) with the anti-phospho-ERK1/2 antibody. ERK1/2 content within total cell lysates is shown as a control. Typical autoradiographs representative of five independent experiments are shown. *B*, graph representing temporal pattern of ERK2 phosphorylation levels (*P-ERK2*) deduced from quantitative results of five representative experiments obtained from analyses using a Java-based image processing program (Image J), developed by Wayne Rasband (National Institutes of Health, Bethesda, MD). *C*, $^{45}Ca^{2+}$ uptake, cAMP production, and insulin secretion from MIN6 cells were measured after incubation with various glucose concentrations with or without 10 nM GLP-1, as described under "Experimental Procedures." Data are means of 3–5 independent experiments. *D*, *F*, and *H*, Western blot assays of phospho-ERK1/2 in MIN6 cells following 10 nM GLP-1 stimulation for 0, 5, or 20 min, with or without inhibitor treatment. The VDCC antagonist nifedipine (2 μM) was not present during the 2-h KRB stabilization period but was added simultaneously with GLP-1 (*D*). The PKA inhibitor H89 (2 μM) or the PI 3-kinase inhibitor wortmannin (100 nM) were added during the 2-h KRB stabilization period and maintained during the 5- or 20-min GLP-1 stimulation (*F* and *H*). 25 μg of protein lysates were then subjected to 10% SDS-PAGE and immunoblotted with the anti-phospho-ERK1/2 antibody. As a control, total ERK1/2 is shown. Typical autoradiographs representative of 3–5 independent experiments are shown. *E*, *G*, and *I*, *graphs* representing levels of ERK2 phosphorylation deduced from quantitative results of 3–5 representative experiments.

next determined the relative role of each signaling pathways in the kinetic pattern of GLP-1-induced ERK1/2 activation.

A rise in intracellular calcium concentration resulting from extracellular calcium influx through the opening of VDCC is known to activate the ERK1/2 pathway (18, 19, 33, 38). We observed that GLP-1 alone has no effect on extracellular calcium influx (Fig. 1*C*). As a control, we verified that GLP-1 was efficient in potentiating 10 mM glucose-induced calcium influx and insulin secretion (Fig. 1*C*). In line with the fact that GLP-1 does not affect basal calcium concentration (Fig. 1*C*), we observed that levels of ERK1/2 phosphorylation induced by GLP-1 at 5 and 20 min were not significantly inhibited by the addition of the VDCC blocker nifedipine (Fig. 1, *D* and *E*). This indicates that, in the absence of glucose, GLP-1 has the capacity to activate ERK1/2 in β-cells independently from an influx of calcium, as previously proposed using the INS-1 β-cell line (19). As a control, we verified that nifedipine blocked the 10 mM glucose-induced calcium influx and insulin secretion (data not shown).

The role of the $G\alpha_s$/cAMP/PKA-dependent component in the kinetic pattern of ERK1/2 activation induced by GLP-1 was next investigated using a PKA inhibitor, H89. We verified that GLP-1 significantly induced the production of cAMP (Fig. 1*C*). An elevation of glucose concentration increased cAMP production as previously described in INS-1 cell line (Fig. 1*C*) (19). Interestingly, pretreatment with H89 at a PKA-selective and non-cytotoxic low concentration, resulted in a ~70% decrease in GLP-1-induced ERK1/2 activation at an early time point (5 min), but no inhibition was observed at a longer time point (20 min) (Fig. 1, *F* and *G*). As a control, we verified that the H89 pretreatment completely suppressed glucagon-induced ERK1/2 activation and had no inhibitory effect on epidermal growth factor-induced ERK1/2 activation (data not shown), as reported (36). These results indicate that the early phase of ERK1/2 activation induced by GLP-1 (5 min) is in great part mediated by a PKA-dependent pathway, whereas the late phase of ERK1/2 activation pattern is completely H89-insensitive and PKA-independent. These findings further suggest that additional signaling pathway(s) are involved in these two phases.

It has been described that GLP-1R activates the phosphoinositide 3-kinase through the transactivation of the EGF receptor (7). Because phosphoinositide 3-kinase has been characterized to display some upstream input activating ERK1/2 in several cellular systems (39), we investigated the role of this kinase in ERK1/2 activation induced by GLP-1. As seen in Fig. 1, *H* and *I*, activation of ERK1/2 by GLP-1 remained unaffected after a wortmannin pretreatment, at a dose able to prevent protein kinase B/AKT phosphorylation induced by insulin (data not shown), indicating that phosphoinositide 3-kinase is not involved in the two phases of ERK1/2 activation induced by GLP-1.

A PKA-independent, β-Arrestin 1-dependent Pathway Mediates the Late Phase of ERK1/2 Activation Induced by GLP1—Recent studies point out an activation of ERK1/2 independent of G-proteins. This G-protein-independent pathway occurs after G-protein uncoupling and results from the recruitment of scaffold proteins, such as β-arrestins, which are proteins initially identified for their role in GPCR desensitization and internalization (40–43). In line with this, β-arrestin 1 has been recently found to be involved in the activation of ERK1/2 by GLP-1 in INS-1 cells (23). Hence, we verified the role of β-arrestin 1 in the kinetic pattern of GLP-1-induced ERK1/2 activation after specifically depleting cellular levels of endogenous β-arrestin 1 in MIN6 cells using an siRNA strategy. The use of 100 nM siRNA duplex (siRNA duplex 2) induced an ~80% decrease in β-arrestin 1 protein expression when compared with control siRNA-transfected cells (Fig. 2*A*). No significant changes in the expression of β-arrestin 2, used as internal and loading control, was observed. In control siRNA-transfected cells, the time course of GLP-1-induced ERK1/2 activation was identical to that observed without any siRNA transfection. However, β-arrestin 1 knockdown significantly altered the time course of ERK1/2 activation. In the absence of β-arrestin 1, the 5-min ERK1/2 activation period was reduced by ~30%, whereas the 20-min ERK1/2 activation period was completely eliminated (Fig. 2, *B* and *C*). Taken together, these results indicate that GLP-1 temporally activates ERK1/2 in β-cells by at least two pathways. The large majority of the early ERK1/2 activation (5 min) induced by GLP-1 is mediated via an H89-sensitive PKA-dependent pathway. A PKA-independent, β-arrestin

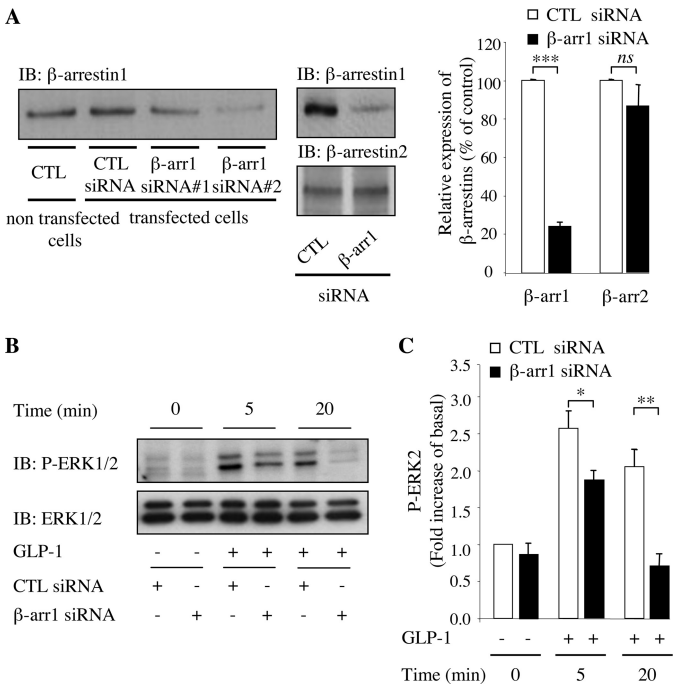

FIGURE 2. **Knockdown of β-arrestin 1 impairs the GLP-1-induced ERK1/2 phosphorylation kinetic pattern in MIN6 cells.** *A*, MIN6 cells were transfected with control or two different β-arrestin 1 siRNA duplexes tested at 25, 50, or 100 nM (final concentrations) for 6 h. 72 h after transfection, cells were lysed, and 35–45 μg of protein lysates were subjected to 10% SDS-PAGE and immunoblotted (*IB*) with anti-β-arrestin 1 and anti-β-arrestin 2 antibodies. The *graph* representing relative expression of β-arrestins following β-arrestin 1 siRNA is deduced from quantitative results of 10 representative experiments. *B*, MIN6 cells were transfected with control or β-arrestin 1 siRNA (*β-arr1 siRNA#2*). Following transfection, cells were incubated in KRB buffer for a 2-h stabilization period and stimulated with 10 nM GLP-1 for various times as indicated. 25 μg of protein lysates were subjected to 10% SDS-PAGE and immunoblotted with the anti-phosphorylated ERK1/2 antibody. ERK1/2 content within total cell lysates is shown as a loading control. Typical autoradiographs representative of eight independent experiments are shown. *C*, the *graph* represents levels of phosphorylation of ERK2 deduced from quantitative results of eight representative experiments. *CTL*, control.

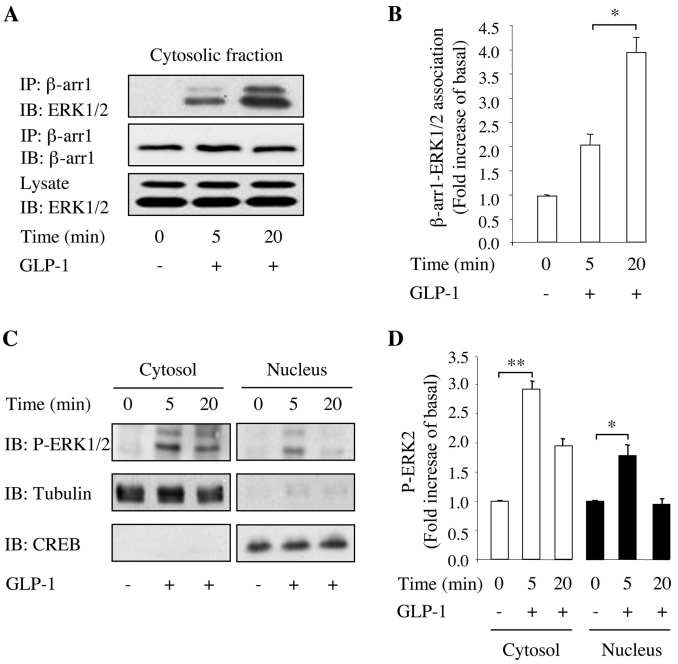

FIGURE 3. **β-Arrestin 1 interacts with ERK1/2 upon GLP-1 stimulation restraining activated ERK1/2 in the cytoplasm.** *A*, after a 2-h stabilization period in KRB buffer, cells were stimulated with 10 nM GLP-1 for various times and lysed, and cytosolic *versus* nuclear fractions were separated by subcellular fractionation. Cytosolic fraction was then subjected to immunoprecipitation (*IP*) in reduced conditions with the mouse monoclonal anti-β-arrestin 1 antibody. Immunoprecipitated proteins were resolved by 10% SDS-PAGE and blotted (*IB*) with anti-total ERK1/2 antibody. Relative quantities of β-arrestin 1 determined by reblots in these immunoprecipitation experiments using the goat polyclonal anti-β-arrestin 1 antibody are also shown. ERK1/2 content within total cell lysates is shown as a control. *B*, the *graph* represents the temporal pattern of β-arrestin 1 and ERK1/2 association deduced from quantitative results of three representative β-arrestin 1 immunoprecipitation experiments. *C*, 2-h KRB-stabilized cells were incubated with 10 nM GLP-1 for various times, as indicated, and nuclear *versus* cytoplasm fractions were prepared. Immunoblots with the active phosphorylated form of ERK1/2 are shown. Tubulin and CREB were also detected as loading control and to show cross-contamination in the cytoplasmic and nuclear fractions, if any. Typical autoradiographs representative of three independent experiments are shown. *D*, the *graph* represents levels of ERK2 phosphorylation deduced from quantitative results of three independent experiments.

1-dependent pathway participates in this early ERK1/2 activation and fully mediates the late activation (20 min) of ERK1/2 induced by GLP1.

Cytosolic Localization of the β-Arrestin 1-dependent ERK1/2 Activation—It is well described that the subcellular localization of activated ERK1/2 is an important factor controlling the access to cytoplasmic *versus* nuclear substrates and thus regulating cell fate decision (21, 22). β-Arrestin 1 is known to interact with protein kinases, such as ERK1/2, leading to their sequestration in the cytoplasmic compartment (44–47). To determine whether there is an association between β-arrestin 1 and ERK1/2 following GLP-1 stimulation in MIN6 cells, immunoprecipitation experiments from cytoplasmic fractions were performed with an anti-β-arrestin 1 antibody. As shown in Fig. 3*A*, no association of β-arrestin 1 with ERK1/2 was found in the basal state. However, we found that GLP-1 induced an association between β-arrestin 1 and ERK1/2 at 5 min, and this interaction strongly increased upon 20-min GLP-1 stimulation (Fig. 3, *A* and *B*). These data demonstrate that ERK1/2 are associated with β-arrestin 1 in the cytoplasmic compartment following GLP-1 stimulation and suggest that this association leads to ERK1/2 sequestration in the cytoplasm.

To gain further insight in functions of ERK1/2 activated by GLP-1 in MIN6 β-cells, we examined their localization by subcellular fractionation at an early (5 min) and late (20 min) time point after GLP-1 stimulation, which represent PKA- and β-arrestin 1-dependent ERK1/2 activation, respectively (Figs. 1*F* and 2*B*). Upon 5-min GLP-1 stimulation, ERK1/2 activation was detected in both the cytoplasm (by about 2.9-fold) and the nucleus (by about 1.8-fold) (Fig. 3, *C* and *D*). Following 20-min GLP-1 stimulation, ERK1/2 activity was found to be exclusively cytoplasmic (Fig. 3, *C* and *D*). These results indicate that upon GLP-1R activation, a pool of ERK1/2 was rapidly activated mainly via the $G\alpha_s$/cAMP/PKA-dependent pathway and found in the β-cell nucleus, where it was rapidly dephosphorylated. By contrast, ERK1/2 activated via the β-arrestin 1 pathway was entirely retained in the cytoplasm. This latter observation suggests that the substrates of ERK1/2 activity elicited by GLP-1 through the β-arrestin 1-mediated signaling pathway may be largely cytoplasmic.

β-Arrestin 1-dependent ERK1/2 Pathway Activates p90RSK—Numerous nuclear substrates have been shown to be phosphorylated by ERK1/2 in β-cells (18, 34, 48–50). However, very little is known about the identity of ERK1/2 cytosolic substrates. Hence, we next examined the potential requirement of ERK1/2 activity in phosphorylation of cytoplasmic targets by GLP-1. p90RSK is a well known downstream kinase for ERK1/2 signaling, activated by phosphorylation in the cytoplasm allowing diverse physiological responses (51, 52). As shown in Fig. 4, *A* and *B*, we found that GLP-1 stimulation leads to slow p90RSK phosphorylation at Thr-573, an important residue for the kinase activation, reaching maximal levels within 10–20 min of GLP-1 treatment and decreasing thereafter. Significant levels of p90RSK phosphorylation were detected in a time period corresponding to the β-arrestin 1-dependent ERK1/2 activation. This latter observation prompted us to ascertain whether phosphorylation of p90RSK induced by GLP-1 is mediated by the β-arrestin 1-dependent ERK1/2 activation pathway. The p90RSK phosphorylation upon GLP-1 stimulation (20 min) was next measured, either in the absence or presence of U0126, a selective inhibitor of MEK1/2, the upstream ERK1/2 kinases. As seen in Fig. 4, *C* and *D*, we found that the phosphorylated active form of p90RSK was totally prevented by the U0126 treatment. As a control, we verified that the U0126 treatment completely abolished the ERK1/2 activation elicited by GLP-1. Moreover, silencing β-arrestin 1 expression by siRNA totally blocked the GLP-1-induced p90RSK phosphorylation (Fig. 4, *E* and *F*). These results demonstrate that GLP-1 phosphorylates and activates p90RSK through the β-arrestin 1-dependent ERK1/2 pathway.

The β-Arrestin 1-dependent ERK1/2-p90RSK Signaling Network Engaged by GLP-1 Mediates the Ser-112 Phosphorylation of Bad—p90RSK is known to have many downstream targets (51, 52). One such target is the cytoplasmic proapoptotic protein Bad, and its phosphorylation by p90RSK has been shown to inhibit its proapoptotic activities (25, 26). Recently, it has been proposed that the phosphorylation state of Bad in β-cells instructs Bad to play a metabolic role by targeting glucokinase

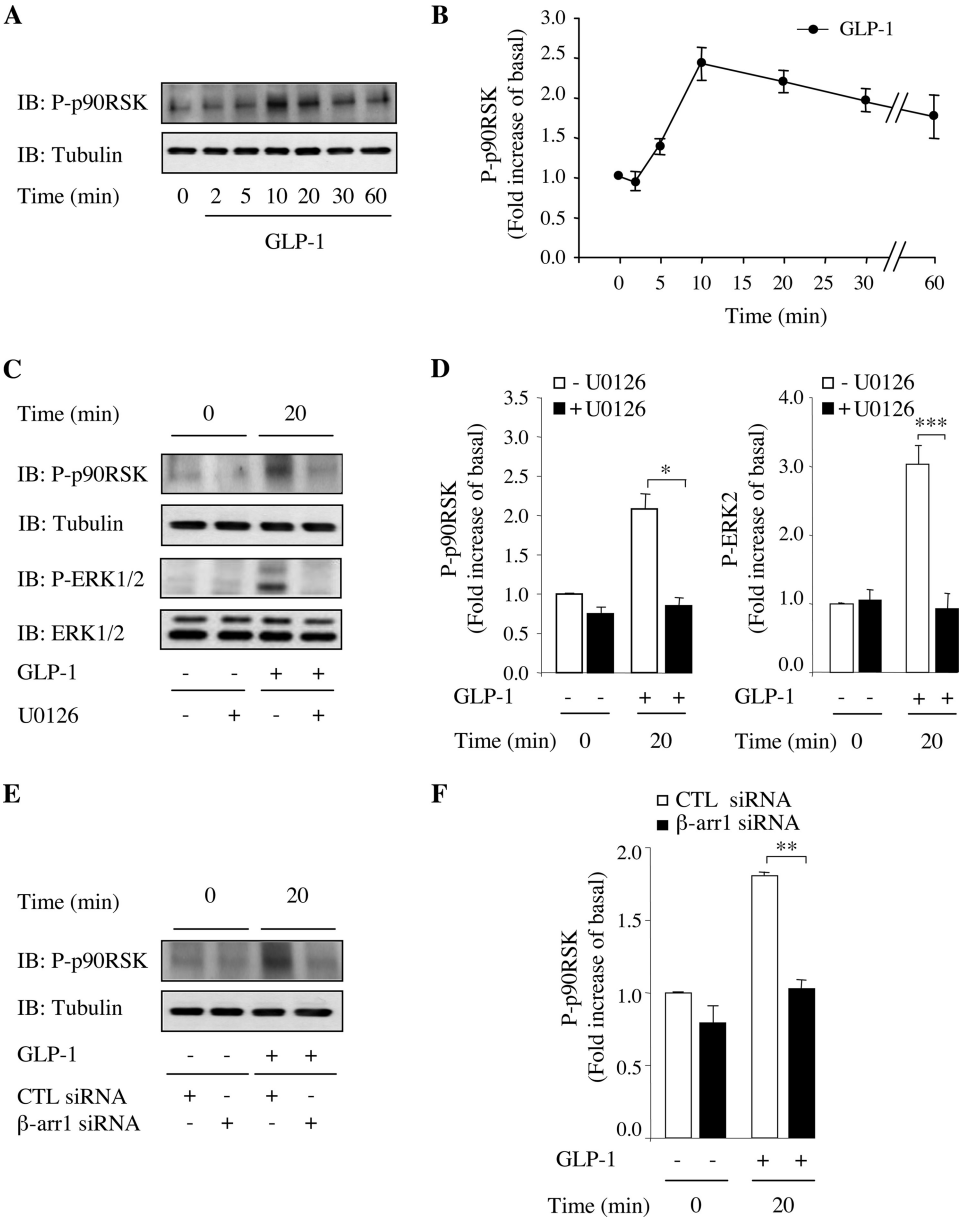

FIGURE 4. **GLP-1 induced p90RSK phosphorylation through a β-arrestin 1-ERK1/2-dependent pathway in MIN6 cells.** *A*, after a 2-h stabilization period in KRB buffer, MIN6 cells were treated with 10 nM GLP-1 for various times as indicated. Identical amounts of lysate proteins (25 μg) were subjected to 10% SDS-PAGE and immunoblotted (*IB*) with the anti-phospho-p90RSK antibody that recognizes the phosphorylated Thr-573 form of this kinase. Tubulin content within total cell lysates is shown as an internal and loading control. Typical autoradiographs representative of five independent experiments are shown. *B*, the *graph* represents levels of phosphorylated p90RSK (*P-p90RSK*) deduced from quantitative results of five representative experiments. *C*, after a 2-h stabilization period in KRB buffer, cells were stimulated with 10 nM GLP-1 for 20 min. The U0126 (10 μM), was added during the 2-h KRB stabilization period and maintained during GLP-1 stimulation. 25 μg of protein lysates were subjected to 10% SDS-PAGE and immunoblotted with anti-phospho-p90RSK or anti-phospho-ERK1/2 antibodies. Tubulin and ERK1/2 contents within total cell lysates are shown as internal and loading control. Typical autoradiographs representative of three independent experiments are shown. *D*, the graphs represent levels of phospho-p90RSK or phospho-ERK2 deduced from quantitative results of three representative experiments. *E*, MIN6 cells were transfected with control or β-arrestin 1 siRNA for 6 h. 72 h after transfection, cells were stabilized in KRB buffer for 2 h and then stimulated for 20 min with 10 nM GLP-1. 25 μg of protein lysates were then subjected to 10% SDS-PAGE and immunoblotted with the anti-phospho-p90RSK antibody. Tubulin content within total cell lysates is shown as a loading control. Typical autoradiographs representative of three independent experiments are shown. *F*, the *graph* represents levels of phospho-p90RSK deduced from quantitative results of three independent experiments. *CTL*, control.

and regulating insulin secretion or alternatively a proapoptotic *versus* an antiapoptotic role (27). The apoptotic activity of Bad can be inhibited by phosphorylation at Ser-112, whereas Bad nucleates a core complex at the mitochondrial membrane containing the glucokinase when phosphorylated at Ser-155 (27). Because phosphorylation at Ser-112 and/or Ser-155 can be mediated by diverse signaling pathways, such as ERK1/2-p90RSK (25, 26), we assessed whether GLP-1 has the capacity to regulate the phosphorylation state-dependent bifunctional role of Bad. Notably, we found that GLP-1 stimulation did not increase Bad phosphorylation at Ser-155, whereas it significantly enhanced the phosphorylation of Bad at Ser-112, which is known to be a preferred p90RSK site (Fig. 5, *A* and *B*). Bad phosphorylation reaches maximal levels slowly (within 20 min of GLP-1 treatment) and remains stable for up to 60 min (Fig. 5, *A* and *B*). Interestingly, a U0126 treatment, which eliminated ERK1/2 and p90RSK activation elicited by GLP-1 (Fig. 4, *C* and *D*), totally suppressed 20-min GLP-1-increased Bad phosphorylation at Ser-112 (Fig. 5, *C* and *D*). Next, we determined whether phosphorylation of Bad at Ser-112 is mediated by the β-arrestin 1-dependent pathway. In control siRNA-transfected cells, GLP-1-induced Bad phosphorylation at Ser-112 is identical to that observed without any siRNA transfection. By contrast, β-arrestin 1 siRNA leads to a complete suppression in GLP-1-mediated Bad phosphorylation (Fig. 5, *E* and *F*). Taken together, these results indicate that the β-arrestin 1-dependent ERK1/2-p90RSK activation engaged by GLP-1 mediates the Ser-112 phosphorylation of Bad.

Bad Is Phosphorylated at Ser-112 through a β-Arrestin 1-mediated ERK1/2-p90RSK Signaling Network in Pancreatic Islets—To confirm, in a more physiological model, the involvement of the ERK1/2-p90RSK signaling network in the GLP-1-induced Bad phosphorylation evidenced in MIN6 cells, we used pancreatic islets isolated from normal control mice (WT). We evaluated the phosphorylation levels of ERK1/2, p90RSK, and Bad induced by GLP-1 in the absence or presence of U0126. In the presence of a low non-insulinotropic glucose concentration (1.4 mM), a 20-min GLP-1 stimulation increased by ~2-fold the

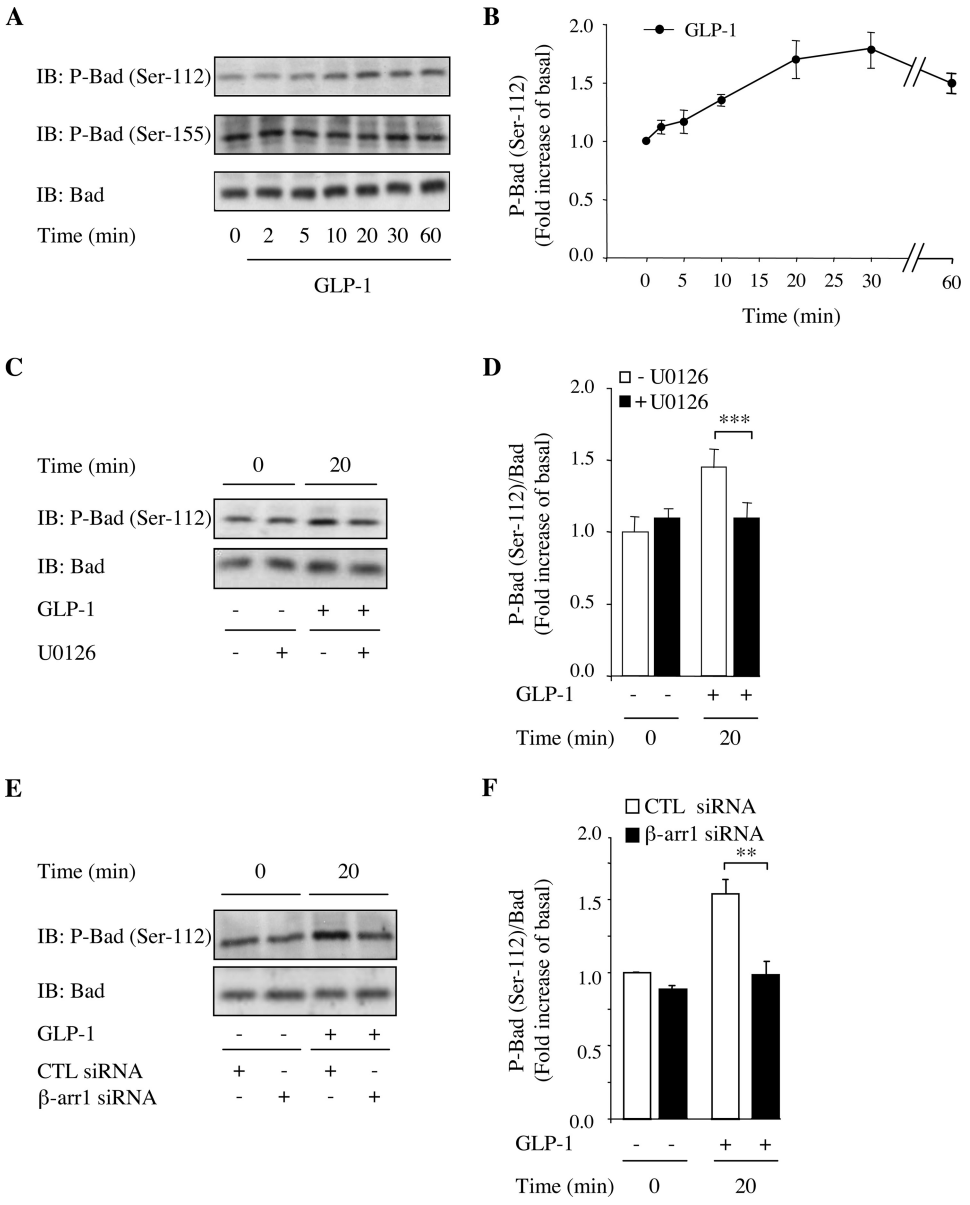

FIGURE 5. **β-Arrestin 1 is required for GLP-1-induced Bad phosphorylation (Ser-112) in MIN6 cells.** *A*, after a 2-h stabilization period in KRB buffer, MIN6 cells were treated with 10 nM GLP-1 for various times as indicated. Identical amounts of lysate proteins (25 μg) were subjected to 10% SDS-PAGE and immunoblotted (*IB*) with the anti-phospho-Bad antibodies that recognize the Ser-112- or the Ser-155-phosphorylated form of the protein. Typical autoradiographs representative of 3–5 independent experiments are shown. Bad content within total cell lysates is shown as internal and loading control. *B*, the *graph* represents levels of phosphorylation of Bad (Ser-112) deduced from quantitative results of 3–5 representative experiments. *C*, after a 2-h stabilization period in KRB buffer, cells were stimulated with 10 nM GLP-1 for 20 min. The U0126 (10 μM) was added during the 2-h KRB stabilization period and maintained during GLP-1 stimulation. 25 μg of protein lysates were subjected to 10% SDS-PAGE and immunoblotted with anti-phospho-Bad (Ser-112) antibody. Bad content within total cell lysates is shown as internal and loading control. Typical autoradiographs representative of three independent experiments are shown. *D*, the graph represents levels of phosphorylation of Bad (Ser-112) deduced from quantitative results of three representative experiments. *E*, MIN6 cells were transfected with control or β-arrestin 1 siRNA for 6 h. 72 h after transfection, cells were stabilized in KRB buffer for 2 h and then stimulated for 20 min with 10 nM GLP-1. 25 μg of protein lysates were then subjected to 10% SDS-PAGE and immunoblotted with the anti-phospho-Bad (Ser-112) antibody. Bad content within total cell lysates is shown as a loading control. Typical autoradiographs representative of three independent experiments are shown. *F*, the *graph* represents levels of phosphorylation of Bad (Ser-112) deduced from quantitative results of three independent experiments. *CTL*, control.

phosphorylation levels of ERK1/2, p90RSK at Thr-573, and Bad at Ser-112 (Fig. 6, *A* and *B*). Fully consistent with the data obtained in MIN6 cells (Figs. 4, *C* and *D*, and 5, *C* and *D*), we observed that ERK1/2, p90RSK, and Bad phosphorylation induced by GLP-1 was totally abolished in islets treated with U0126 (Fig. 6, *A* and *B*). It is noteworthy that under low non-insulinotropic glucose concentration, ERK1/2 and p90RSK are phosphorylated within the islets and can be inhibited by treatment with ERK1/2 inhibitor U0126 (Fig. 6, *A* and *B*). Interestingly, the U0126 treatment did not inhibit the basal Ser-112 phosphorylation of Bad, suggesting that under non-insulinotropic glucose concentration, the Ser-112 residue of Bad is phosphorylated within the islets independently from the ERK1/2-p90RSK signaling network (Fig. 6, *A* and *B*). We further investigated the biological significance of β-arrestin 1 on GLP-1 signaling using pancreatic islets isolated from WT and β-arrestin 1 KO mice (31). In the presence of a low non-insulinotropic glucose concentration, a GLP-1 stimulation enhanced the phosphorylation of ERK1/2 and significantly induced Bad phosphorylation at Ser-112 (by about 1.8-fold; Fig. 6, *C* and *D*) in WT mouse islets. In contrast, we observed that ERK1/2 activation and Bad phosphorylation induced by GLP-1 were totally abolished in β-arrestin 1 KO mouse islets (Fig. 6, *C* and *D*). Taken together, these results confirm that GLP-1 phosphorylates Bad at Ser-112 through a β-arrestin 1-mediated ERK1/2-p90RSK signaling network activation in pancreatic islets.

β-Arrestin 1 Plays a Key Role in the Binding of Bad to the Scaffold Protein 14-3-3 Induced by GLP-1—It is well described that phosphorylation of Bad at Ser-112 allows binding of Bad to the scaffold protein 14-3-3, preventing its proapoptotic functions. Therefore, we investigated whether GLP-1 induces the interaction of Bad with the 14-3-3 protein and whether this is mediated by β-arrestin 1 signaling. As shown in Fig. 7, *A* and *B*, the immunoprecipitation experiments with Bad antibody followed by immunoblotting for 14-3-3 protein showed a slight interaction between Bad and 14-3-3 protein in the basal control state, whereas a marked and significant association was observed following a 20-min GLP-1 stimulation. We observed that this interaction

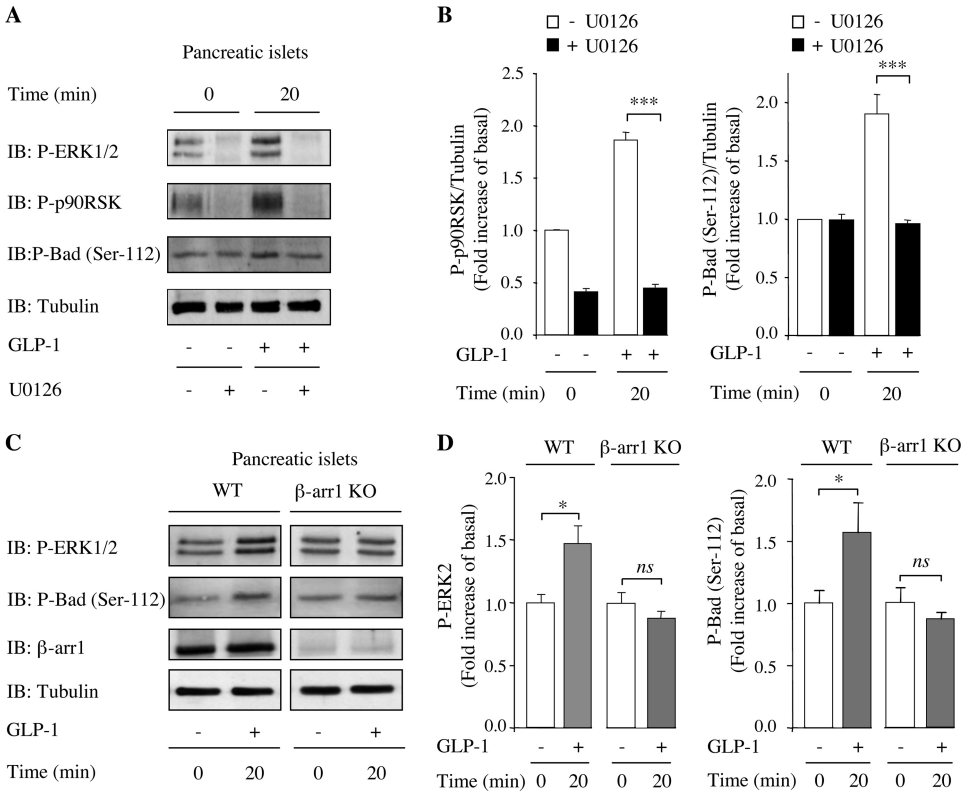

FIGURE 6. **β-Arrestin 1 is required for GLP-1-induced ERK1/2 and Bad phosphorylation (Ser-112) in mouse pancreatic islets.** *A*, islets isolated from normal control mice were stabilized in KRB buffer supplemented with 1.4 mM glucose for 2 h and then incubated with 1.4 mM glucose plus 100 nM GLP-1 for 20 min. The U0126 (10 μM) was added during the 2-h KRB stabilization period and maintained during GLP-1 stimulation. 25 μg of protein lysates were subjected to 10% SDS-PAGE and immunoblotted (*IB*) with the anti-phospho-ERK1/2, anti-phospho-p90RSK, anti-phospho-Bad (Ser-112), or anti-tubulin antibodies. The most representative blots obtained from three independent experiments are shown. *C*, islets isolated from WT or β-arrestin 1 KO mice were stabilized in KRB buffer supplemented with 1.4 mM glucose for 2 h and then incubated with 1.4 mM glucose plus 100 nM GLP-1 for 20 min. 15–25 μg of protein lysates were subjected to 10% SDS-PAGE and immunoblotted with the anti-phospho-ERK1/2, anti-phospho-Bad (Ser-112), anti-β-arrestin 1, or anti-tubulin antibodies. The most representative blots obtained from three independent experiments performed with 10–12 WT or β-arrestin 1 KO mice are shown. *B* and *D*, the *graphs* represent levels of phosphorylation of ERK2, p90RSK, and phosphorylation of Bad (Ser-112) deduced from quantitative results of three independent experiments.

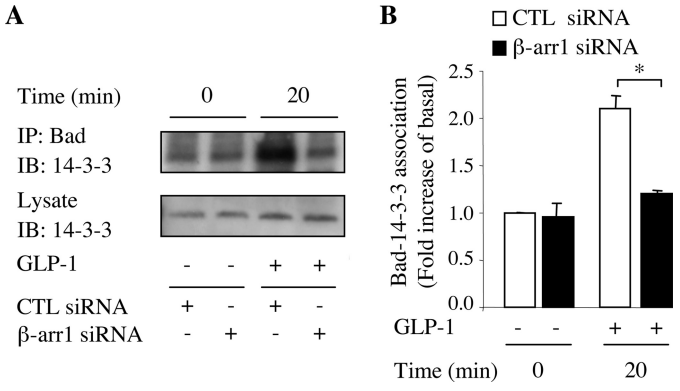

FIGURE 7. **β-Arrestin 1 knockdown prevents the interaction of BAD with the 14-3-3 protein induced by GLP-1 in MIN6 cells.** *A*, MIN6 cells were transfected with control or β-arrestin 1 siRNA for 6 h. 72 h after transfection, cells were stabilized in KRB buffer for 2 h and then stimulated for 20 min with 10 nM GLP-1. Cell lysates were then subjected to immunoprecipitation (*IP*) in reduced conditions with the mouse monoclonal anti-Bad (C7) antibody. Immunoprecipitated proteins were resolved by 10% SDS-PAGE and blotted (*IB*) with anti-total 14-3-3 β (C-20) protein antibody. 14-3-3 protein content within total cell lysates is shown as control. *B*, the *graph* represents the association of Bad with 14-3-3 protein deduced from quantitative results of three representative Bad immunoprecipitation experiments. *CTL*, control.

was totally prevented in β-arrestin 1 siRNA-transfected cells (Fig. 7, *A* and *B*), demonstrating that the activation of GLP-1R leads to a β-arrestin 1-dependent increase in Bad interaction with 14-3-3 protein.

β-Arrestin 1 and Ser^{112} Phosphorylation of Bad Mediate GLP-1 Anti-apoptotic Effect—We further investigated the role of β-arrestin 1 in regulating apoptosis. Apoptosis was detected in MIN6 cells by evaluating levels of 17-kDa cleaved caspase-3, a key executioner and marker of apoptosis (53). Prolonged culture of β-cells (>48 h) in optimal glucose concentrations (6–10 mM) promotes their survival, whereas prolonged culture in supraoptimal glucose concentrations (>10 mM) induces apoptosis (34, 54, 55). Culture in serum-free DMEM containing 10 mM glucose protected MIN6 cells from apoptosis because any emergence of cleaved caspase-3 was noticed over 72 h (Fig. 8, *A* and *B*). In contrast, exposure of MIN6 cells to serum free-DMEM containing a high glucose concentration (25 mM) for 72 h led to apoptosis and increased cleaved caspase-3 (Fig. 8, *A* and *B*). These observations are consistent with previous reports (34, 54, 55) demonstrating that the viability of cultured β-cells and isolated islets depends on the prevailing glucose concentration, with an optimal glucose concentration for rodent β-cell survival around 10 mM. Depletion of β-arrestin 1 cellular content by siRNA did not cause the emergence of cleaved caspase-3 in MIN6 cells cultured in 10 mM glucose for 72 h (Fig. 8, *A* and *B*). To further validate these findings, we measured DNA fragmentation, a feature of cell death, in pancreatic islets isolated from WT and β-arrestin 1 KO mice. Consistent with the results obtained from MIN6 cells transfected with β-arrestin 1 siRNA, no increases in DNA fragmentation and apoptotic cell death were noticed in β-arrestin 1 KO islets cultured in optimal glucose concentration for survival (*i.e.* 10 mM glucose) compared with WT islets (Fig. 8*C*). Taken together, these results suggest that β-arrestin 1 does not play a role in β-cell survival mediated by optimal glucose concentration. However, because we observed that the β-arrestin 1 KO islets have a significant defective glucose-stimulated insulin secretion (Fig. 9*A*), our results further indicate that β-arrestin 1 plays a key role in β-cell function. Insulin content and total cellular protein content were not affected by β-arrestin 1 knockdown (Fig. 9, *B* and *C*), suggesting that the effects on insulin secretion were not attributed to a decrease in insulin availability or cellular protein expression.

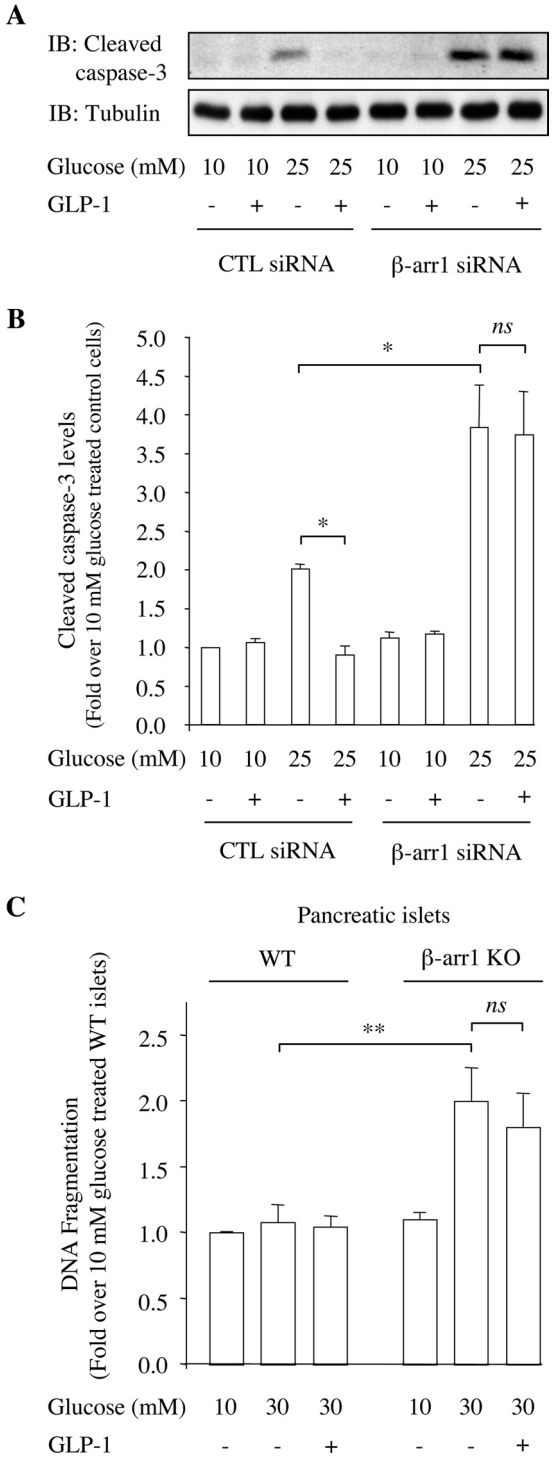

FIGURE 8. **Role of β-arrestin 1 in GLP-1-stimulated protection from apoptotic β-cell death.** *A*, MIN6 cells were transfected with control or β-arrestin 1 siRNA. Following transfection, cells were cultured in serum free-DMEM containing 10 or 25 mM glucose in the presence or absence of 10 nM GLP-1 for 72 h. 45 μg protein lysates were subjected to 12.5% SDS-PAGE and immunoblotted (*IB*) with the anti-cleaved caspase-3 or anti-tubulin antibodies. Tubulin content within total cell lysates is shown as a loading control. Typical autoradiographs representative of 5 independent experiments are shown. *B*, the *graph* represents levels of cleaved caspase-3 deduced from quantitative results of five representative experiments. *C*, following isolation, islets were cultured overnight in RPMI containing 10 mM glucose and 5% bovine serum albumin. DNA fragmentation was measured in islets cultured in RPMI containing 10 or 30 mM glucose in the presence or absence of 100 nM GLP-1 for an additional 120-h period. The graph represents levels of DNA fragmentation deduced from quantitative results of 3–5 representative experiments. *CTL*, control.

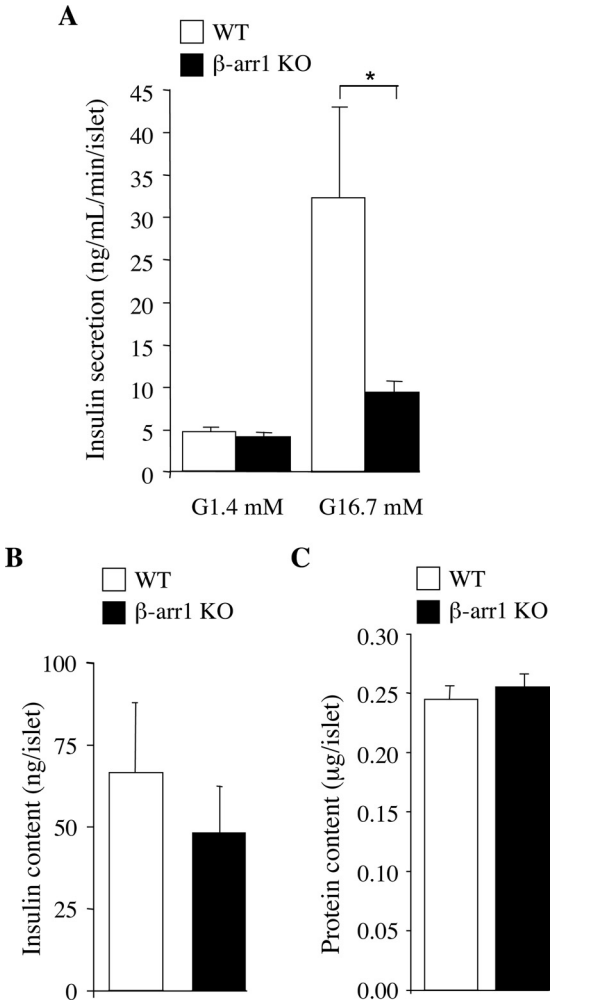

FIGURE 9. **Glucose-stimulated insulin secretion from WT or β-arrestin 1 KO mice.** *A*, islets were preincubated for 30 min in KRB buffer supplemented with 1.4 mM glucose. Following preincubation, islets were further stimulated with 1.4 or 16.7 mM glucose for 20 min. Data are means of five independent experiments. *B* and *C*, insulin and protein content determination as described under "Experimental Procedures."

We further investigated the potential requirement of β-arrestin 1 in the protective effect of GLP-1 against apoptosis elicited by chronic high glucose exposure. We first evaluated the effects of GLP-1 on the emergence of cleaved caspase-3 induced by prolonged high glucose exposure in MIN6 cells transfected with either control or β-arrestin 1 siRNA. As shown in Fig. 8, *A* and *B*, the emergence of cleaved caspase-3 induced by this apoptotic situation was blocked by GLP-1 treatment in control siRNA-transfected cells. Depletion of β-arrestin 1 by siRNA not only increases cleaved caspase-3 levels induced by the prolonged high glucose exposure up to ~1.8-fold but also eliminates the GLP-1-mediated decrease in these levels (Fig. 8, *A* and *B*). Interestingly, treatment of WT islets with high glucose concentration (30 mM) for 120 h did not cause any increase in DNA fragmentation (Fig. 8*C*). This observation is fully consistent with previous studies reporting a lack of sensitivity to long term high glucose toxicity in islets isolated from the C57BL/6J mouse strain (56, 57). In contrast, treatment of β-arrestin 1 KO islets with a 30 mM glucose concentration for 120 h caused a ~2-fold increase in DNA fragmentation. Notably, although GLP-1 was

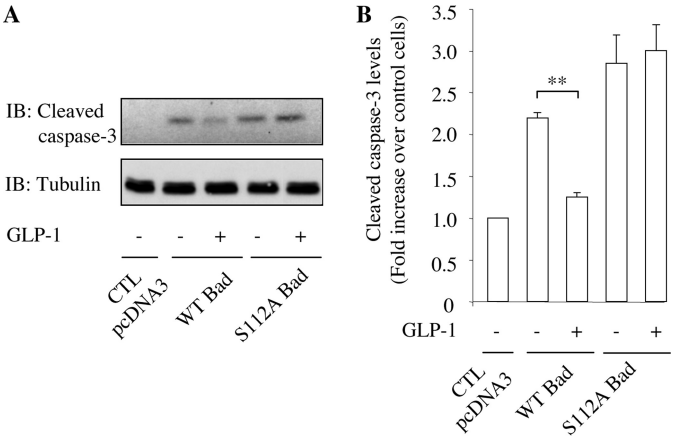

FIGURE 10. **p90RSK phosphorylation of Bad is required for GLP-1 antiapoptotic effect.** *A*, MIN6 cells were transfected with control pcDNA3 vector, WT Bad, or mutant Bad S112A for 6 h. Following transfection, cells were cultured in serum-free DMEM containing 10 mM glucose in the presence or absence of 10 nM GLP-1 for 72 h. Levels of cleaved caspase-3 and tubulin were visualized by immunoblotting (*IB*). Tubulin content within total cell lysates is shown as loading control. Typical autoradiographs representative of three independent experiments are shown. *B*, the *graph* represents levels of cleaved caspase-3 deduced from quantitative results of three independent experiments. *CTL*, control.

found to be active in phosphorylating ERK1/2 and Bad in WT islets, it failed to activate the ERK1/2-Bad signaling network in β-arrestin 1 KO islets (Fig. 6, *C* and *D*) and to decrease the emergence of DNA fragmentation in high glucose treated-β-arrestin 1 KO islets (Fig. 8*C*). This latter observation is similar to the situation observed with cleavage of caspase-3 induced by high glucose in MIN6 cells (Fig. 8*A*). Collectively, these results indicate the importance of β-arrestin 1 in GLP-1-stimulated protection from apoptotic cell death induced by chronic high glucose exposure. These data also suggest that maintenance of β-arrestin 1 protein expression in β-cells participates in the protection against the emergence of glucotoxicity.

We finally assessed whether ERK1/2-p90RSK-mediated phosphorylation of Bad is required for the antiapoptotic effect of GLP-1 in β-cells. To address this issue, we used a mutant plasmid, Bad S112A, that is defective in p90RSK-mediated phosphorylation (25) and evaluated the emergence of cleaved caspase-3 in MIN6 cells overexpressing the proapoptotic WT Bad or the mutant Bad S112A. As shown in Fig. 9, *A* and *B*, increases in cleaved caspase-3 levels were observed in both situations (*i.e.* WT Bad and mutant Bad S112A). Notably, we observed that GLP-1 treatment led to ~70–80% decreases in cleaved caspase-3 levels in cells overexpressing the WT Bad, whereas such reduction was not observed in cells overexpressing the mutant Bad S112A (Fig. 10, *A* and *B*). These results indicate that p90RSK phosphorylation of Bad is required for β-arrestin 1-mediated GLP-1 antiapoptotic effect.

DISCUSSION

Strategies based on activating GLP-1R are intensively developed for the treatment of type 2 diabetes. The knowledge of the signaling pathways linked to activated GLP-1R within β-cells and a better understanding of their functions are of major significance. ERK1/2 is involved in many cellular functions, such as proliferation, differentiation, and survival. The cellular effects mediated by ERK1/2 depend on their spatial and temporal activation patterns (21, 22). In β-cells, GLP-1 has been reported to activate ERK1/2 via diverse pathways (17–20, 23). However, the impact of these various pathways on the temporal and subcellular distribution of ERK1/2 activation remains totally unknown. Here, we report that GLP-1 activates ERK1/2 via different temporal components in β-cells. One occurs early and mainly depends on PKA activation, leading to the translocation of activated ERK1/2 in the nucleus, whereas the other has a slower and late onset and is completely β-arrestin 1-dependent, restraining the activated kinases in the cytoplasm. Supporting the concept that the ERK1/2 activation is a significant regulator of GLP-1-dependent cytoplasmic actions, we further demonstrate that the β-arrestin 1-dependent ERK1/2 signaling engaged by GLP-1 stimulates p90RSK activity mediating the phosphorylation of Bad at Ser-112 and regulating its binding to the scaffold protein 14-3-3. The β-arrestin 1-ERK1/2/p90RSK signaling mediates GLP-1 antiapoptotic effect by regulating Bad phosphorylation at Ser-112.

We first found that GLP-1 induced a two-phase kinetic pattern of ERK1/2 activation, with a rapid and transient first phase followed by a late second phase remaining stable for up to 60 min. Interestingly, we showed recently that another class II GPCR, PACAP receptor (PAC1 receptor), when activated in the absence of insulinotropic glucose concentration, induces only a transient first phase of ERK1/2 activation in β-cells (59), indicating differences between these two GPCRs in their abilities to engage temporally distinct signaling pathways leading to ERK1/2 activity.

The role of PKA in the regulation of ERK1/2 activation by GLP-1 in β-cells remains controversial (17–19). We found that only the early phase of ERK1/2 activity induced by GLP-1 is in great part mediated by a PKA-dependent pathway, whereas the late phase of ERK1/2 activation pattern is completely PKA-independent. In many cells, cAMP-PKA and intracellular $[Ca^{2+}]$ second messengers are tightly coupled. Increased intracellular $[Ca^{2+}]$ has been shown to stimulate the ERK1/2 signaling cascade (18, 19, 33, 38). It is likely that GLP-1-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 the situation of non-insulinotropic glucose concentration, GLP-1 failed to induce calcium influx, whereas PKA is active. However, we cannot rule out the possibility that GLP-1 induces a rise in intracellular $[Ca^{2+}]$ that results from mobilization of intracellular pools of calcium (and not from extracellular calcium influx), most probably via cAMP production or PKA activation, participating in the activation of ERK1/2.

We report that the β-arrestin 1-dependent pathway fully mediates the late activation of ERK1/2 induced by GLP-1. β-Arrestin 1 has been first described as a mediator for GPCR desensitization and internalization (40, 41). We found that β-arrestin 1 depletion by siRNA reduced but did not enhance the 5-min cAMP/PKA-dependent ERK1/2 activation, suggesting that β-arrestin 1 is not involved in a GLP-1R-$G\alpha_s$ desensitization mechanism, which is consistent with the findings of Sonoda *et al.* (23). Now, our data raise the question of how β-arrestin 1 couples the GLP-1R to the ERK1/2 cascade. It is

well described that β-arrestin 1 can mediate G protein-independent signaling by acting to scaffold a variety of proteins, such as ERK1/2 (40–44). It has been reported that β-arrestin 1 physically interacts with the GLP-1R without affecting the internalization process of the receptor (23, 60). In this study, we observed an association between β-arrestin 1 and ERK1/2 upon GLP-1 stimulation, suggesting that β-arrestin 1 might function as a scaffold protein bringing ERK1/2 to the activated GLP-1R. By interacting with the GLP-1R, β-arrestin 1 may provide a scaffold platform to dock other proteins to the receptor, allowing ERK1/2 activation to proceed, as observed for several GPCRs, such as the angiotensin II type 1A, neurokinin 1, and protease-activated receptors (45, 61, 62). It is now of particular interest to investigate the molecular mechanisms regulating the spatiotemporal formation of the β-arrestin 1-ERK1/2 complex upon GLP-1 stimulation.

Recent data suggest that the different pathways leading to ERK1/2 activation downstream of GPCRs are mechanistically distinct and exert different signaling functions. Hence, whereas G-protein dependent ERK1/2 activation is transient and nuclear, ERK1/2 activation through the β-arrestin-dependent pathway is sustained and remains in the cytosol (45–47). Consistent with this, we found that the ERK1/2 activation induced by GLP-1 through cAMP/PKA and/or β-arrestin 1 pathways is not only temporally but also spatially segregated. GLP-1 mediates rapid and transient ERK1/2 activation through the $G\alpha_s$/cAMP/PKA pathway, leading to the nuclear translocation of these kinases. This nuclear pool of active ERK1/2 may provide a transcriptional response and/or a mitogenic stimulus by directly activating transcription factors, such as Beta2/NeuroD1, MafA, or the ETS family member Elk-1 (8, 22, 48, 63). By contrast, ERK1/2 activation mediated by β-arrestin 1 spatially constrains ERK1/2 activity in the β-cell cytoplasm and favors the phosphorylation of non-nuclear ERK1/2 substrates. Thus, one function of β-arrestin 1-bound ERK1/2 may be to direct the kinases toward extranuclear substrates involved in translation, cell survival, or cytoskeletal rearrangement and not directly involved in transcriptional control. In line with this, we found that the β-arrestin 1-dependent ERK1/2 activity leads to the activation of p90RSK mediating the phosphorylation of the cytoplasmic proapoptotic protein Bad. However, we cannot rule out the possibility that a pool of p90RSK activated by the β-arrestin 1-ERK1/2 pathway translocates to the nucleus and in turn relays signals. Hence, it can be hypothesized that the β-arrestin 1-mediated ERK1/2 activation might also regulate a specific pattern of gene transcription that exerts different cellular functions from those associated with the classical transcriptional regulation induced by nuclear ERK1/2.

Cytoplasmic ERK1/2 retention may affect the duration of ERK1/2 signaling. Indeed, evidence suggests that the nucleus represents a site not only for ERK1/2 action but also for ERK1/2 signal termination because these kinases can be dephosphorylated by nuclear ERK1/2 phosphatases, such as MKP1 to -3 (64). Consistent with this, we observed that the nuclear pool of ERK1/2 activated by GLP-1 at 5 min via the main cAMP/PKA-dependent pathway was rapidly dephosphorylated. By constraining long lasting ERK1/2 activity in the β-cell cytoplasm, β-arrestin 1-dependent pathway may allow persistent ERK1/2 activation and phosphorylation of cytosolic substrates.

It has been shown that the phosphorylation state of Bad within the β-cells instructs Bad to regulate insulin secretion or alternatively to play a proapoptotic *versus* an antiapoptotic role (27, 65). In the absence of insulinotropic glucose concentration, we found that activated GLP-1R does not stimulate insulin secretion, as expected, whereas it leads to the phosphorylation of the proapoptotic protein Bad at Ser-112, through the β-arrestin 1-dependent ERK1/2-p90RSK signaling network, targeting Bad to the scaffold protein 14-3-3 and inducing its inactivation. We further observed that the ERK1/2-p90RSK-mediated phosphorylation of Bad is required for β-arrestin 1-mediated GLP-1 antiapoptotic effect. Thus, based on our results, this new regulatory mechanism engaged by activated GLP-1R is now suspected to participate in the maintenance of β-cell survival. When phosphorylated at Ser-155, it has been shown that Bad aggregates a molecular complex at the mitochondrial membrane containing the glucokinase and thus plays a pivotal role in glucose-induced insulin secretion (27). In the absence of insulinotropic glucose concentration, we found that GLP-1 did not phosphorylate Bad at Ser-155. However, it can be hypothesized that, in the presence of insulinotropic glucose concentration and massive intracellular $[Ca^{2+}]$ increase due to calcium influx through the opening of VDCCs, Bad undergoes conformational changes. This might allow GLP-1 to phosphorylate the residue Ser-155, favoring the location of Bad within the glucokinase complex for the potentiation of glucose-induced insulin secretion. The fact that GLP-1 differentially controls the phosphorylation state of Bad, instructing Bad to regulate insulin secretion or to play an antiapoptotic role in various glucose concentrations, is an attractive working hypothesis and deserves now to be investigated in detail.

Finally, it should be noted that activation of the angiotensin II type 1A, a class I GPCR, has been recently found to induce the phosphorylation of ERK1/2 and Bad through a β-arrestin 2-dependent signaling (66, 67). Here, we found that activation of the GLP-1R, a class II GPCR, phosphorylates ERK1/2 and Bad through a β-arrestin 1-dependent signaling. Our results highlight that the angiotensin II type 1A and the GLP-1R belonging to distinct GPCR classes differ in their capacities to engage a specific β-arrestin isoform as a critical component of a signaling network regulating ERK1/2 cascade, Bad inactivation, and apoptosis.

In conclusion, we demonstrate that GLP-1, via different pathways, induces the formation of functionally distinct pools of active ERK1/2 in β-cells. The preservation of a functional β-cell mass has become a major point of research, and the future therapy of type 2 diabetes aims at protecting the β-cell from apoptotic death. Because of its therapeutic utility in the treatment of type 2 diabetes, drug discovery has focused on enhancing GLP-1 β-cell actions (1–5). GLP-1 analogues are currently tested for their capacity to engage G-protein activity, cAMP production, and PKA activation. In this study, we found that the long lasting ERK1/2 activation is independent of PKA and is mediated through the β-arrestin 1 pathway leading, via p90RSK activation, to the inactivation of the proapoptotic protein Bad and to the protection against apoptosis. Our study

encourages the screening of these molecules not only for their ability to mediate G-protein signaling but also for their capacity to engage the β-arrestin 1 pathway. This β-arrestin 1 pathway may serve as a prototype to generate new therapeutic GLP-1 agents. As performed for other GPCRs (58, 68), development of GLP-1R ligands that specifically activate the β-arrestin 1 pathway (biased ligands) should facilitate a greater understanding of the physiological relevance of this new signaling pathway.

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