Glucagon-like Peptide-2 Receptor Activation Engages Bad and Glycogen Synthase Kinase-3 in a Protein Kinase A-dependent Manner and Prevents Apoptosis following Inhibition of Phosphatidylinositol 3-Kinase*

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Activation of glucagon-like peptide-2 receptor (GLP-2R) signaling promotes expansion of the mucosal epithelium indirectly via activation of growth and anti-apoptotic pathways; however, the cellular mechanisms coupling direct GLP-2R activation to cell survival remain poorly understood. We now demonstrate that GLP-2, in a cycloheximide-insensitive manner, enhanced survival in baby hamster kidney cells stably transfected with the rat GLP-2R; reduced mitochondrial cytochrome *c* efflux; and attenuated the caspase-dependent cleavage of Akt, poly(ADP-ribose) polymerase, and β-catenin following inhibition of phosphatidylinositol 3-kinase (PI3K) by LY294002. The prosurvival effects of GLP-2 on LY294002-induced cell death were independent of Akt, p90[Rsk], or p70 S6 kinase activation; were mimicked by forskolin; and were abrogated by inhibition of protein kinase A (PKA) activity. GLP-2 inhibited activation of glycogen synthase kinase-3 (GSK-3) through phosphorylation at Ser[21] in GSK-3α and at Ser[9] in GSK-3β in a PI3K-independent, PKA-dependent manner. GLP-2 reduced LY294002-induced mitochondrial association of endogenous Bad and Bax and stimulated phosphorylation of a transfected Bad fusion protein at Ser[155] in a PI3K-independent, but H89-sensitive manner, a modification known to suppress Bad pro-apoptotic activity. These results suggest that GLP-2R signaling enhances cell survival independently of PI3K/Akt by inhibiting the activity of a subset of pro-apoptotic downstream targets of Akt in a PKA-dependent manner.

The endocrine pancreas and intestinal endocrine system produce peptide hormones that regulate food intake, gastrointestinal motility, acid secretion, nutrient transit, and both nutrient absorption and disposal. The majority of these actions are rapid, occur within minutes following activation of distinct hormone-specific G protein-coupled receptors, and serve to modulate the intake and assimilation of energy in both the fasting and postprandial states.

The proglucagon gene is expressed in both the endocrine pancreas and intestine and, following tissue-specific processing of proglucagon, gives rise to multiple peptides, including glucagon in the endocrine pancreas and glucagon-like peptide-1 (GLP-1),[1] and glucagon-like peptide-2 (GLP-2) in the intestine (1). Glucagon acts to maintain energy homeostasis through the hepatic control of glycogenolysis and gluconeogenesis and serves as the primary counter-regulatory hormone that opposes insulin action and thereby prevents hypoglycemia (2). In contrast, GLP-1 enhances the disposal of ingested nutrients via inhibition of glucagon secretion and stimulation of insulin secretion from the islet β cell. The acute metabolic actions of GLP-2 are less well understood; however, exogenous administration of GLP-2 inhibits gastric acid secretion and gastric motility, reduces intestinal permeability, and enhances intestinal hexose transport in rodents *in vivo* (3).

The most striking consequence of GLP-2 action is expansion of the small bowel mucosal epithelium. GLP-2 administration stimulates crypt cell proliferation, increases crypt and villus height, and augments mucosal surface area in both rats and mice (4, 5). Intriguingly, GLP-1 also exerts trophic effects in the endocrine pancreas, including stimulation of islet ductal neogenesis and β cell proliferation in normal rodents *in vivo* and in β cell lines *in vitro* (6, 7). These actions of GLP-1 and GLP-2 are preserved in experimental models of diabetes and intestinal disease, respectively; hence, activation of glucagon-like peptide receptor signaling pathways leads to enhanced cell proliferation in both normal and injured tissues *in vivo*.

More recent data suggest that the glucagon-like peptides exert direct cytoprotective effects via inhibition of apoptosis either directly in target cells expressing their cognate receptors or indirectly via liberation of as yet unidentified survival factors. GLP-1 inhibits apoptosis in islet β cells or in heterologous baby hamster kidney (BHK) fibroblasts expressing a transfected GLP-1 receptor (8, 9). GLP-2 administration to rodents with experimental intestinal injury markedly attenuates mucosal damage and significantly reduces the extent of apoptosis in both the crypt and enterocyte compartments (10, 11).

As enriched populations of intestinal cells or cell lines that express the endogenous GLP-2 receptor (GLP-2R) have not yet been isolated and characterized *in vitro*, we established a cel-

[1] The abbreviations used are: GLP, glucagon-like peptide; GLP-2R, GLP-2 receptor; rGLP, rat GLP; BHK, baby hamster kidney; PMA, phorbol 12-myristate 13-acetate; PI3K, phosphatidylinositol 3-kinase; GSK-3, glycogen synthase kinase-3; ERK, extracellular signal-regulated kinase; PKA, protein kinase A; GPCR, G protein-coupled receptor.

lular model for analysis of GLP-2R signal transduction *in vitro*. BHK fibroblasts stably transfected with the rat GLP-2R exhibit dose-dependent cAMP accumulation in response to GLP-2 administration (12). Remarkably, induction of apoptosis in BHK-rGLP-2R cells with cycloheximide is markedly attenuated by GLP-2, in association with reduced mitochondrial cytochrome *c* efflux to the cytosol and diminished cleavage and activation of both initiator and effector caspases (13). To understand the mechanisms stimulated by GLP-2R activation that couple cAMP accumulation to inhibition of cell death, we examined signaling molecules that represent potential anti-apoptotic targets following activation of the G protein-coupled GLP-2R.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture medium, serum, and G418 were from Invitrogen. Cycloheximide, forskolin, phorbol 12-myristate 13-acetate (PMA), protease inhibitor mixture (P-2714), and phosphatase inhibitor mixture I were purchased from Sigma. Recombinant human [Gly^2]GLP-2 (hereafter abbreviated GLP-2) was a kind gift from NPS Allelix Inc. (Mississauga, Canada). Recombinant human insulin was from Lilly. The pan-caspase inhibitor benzyloxycarbonyl-VAD-fluoromethyl ketone and the kinase inhibitors H89 and LY294002 were obtained from Calbiochem. All electrophoresis reagents were purchased from Bio-Rad. The expression vector MtR(AB) (16) was a gift from G. S. McKnight.

Cell Culture, Apoptosis Induction, and Drug Treatments—BHK fibroblasts containing the stably integrated pcDNA3.1 plasmid (Invitrogen) directing expression of the rat GLP-2R (BHK-rGLP-2R cells) were propagated as described previously (12). When used for experiments, cells were plated in culture medium lacking G418. Upon reaching 70–80% confluence, the cultures were maintained for 15–17 h in serum-depleted medium (Dulbecco's modified Eagle's medium supplemented with 0.1% calf serum) prior to apoptosis induction by the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 in the same medium in the presence or absence of the indicated peptides or drugs for the indicated periods of time. Wortmannin, another widely used PI3K inhibitor, was as effective as LY294002 for transiently preventing serum-induced phosphorylation of Akt at Ser^{473}, a marker of PI3K activation. However, unlike LY294002, the inhibition of Akt Ser^{473} phosphorylation by wortmannin (even at 1 μM) was not sustained and disappeared almost completely after 5–6 h of treatment. Consistent with these findings, cell viability was reduced only by ~25% in the presence of wortmannin (data not shown); hence, wortmannin was not used in further experiments.

GLP-2 and insulin were dissolved in phosphate-buffered saline, and benzyloxycarbonyl-VAD-fluoromethyl ketone, forskolin, PMA, H89, and LY294002 were dissolved in dimethyl sulfoxide. Control cultures were subjected to the same manipulations as treated cells, but in the absence of the drugs. Dimethyl sulfoxide final concentration was identical in every culture irrespective of the particular treatment group.

Cell Viability Assay—The number of viable cells remaining following apoptosis induction was assessed by measuring the bioreduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium salt at 490 nm using the CellTiter 96 aqueous assay (Promega, Madison, WI).

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis—Following induction of apoptosis as indicated above, adherent cells were scrapped off the culture dishes, combined with detached cells floating in the medium, and lysed in radioimmune precipitation assay buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in phosphate-buffered saline) supplemented with protease and phosphatase inhibitor mixtures (both at 1:100 dilution), 10 μg/ml pepstatin A, 5 mM sodium fluoride, and 0.5 mM sodium orthovanadate. Cell lysates were cleared at 12,000 rpm for 15 min at 4 °C, boiled for 5 min with sample buffer containing β-mercaptoethanol, and stored at −70 °C until used. Protein concentration was determined using the BCA protein assay (Pierce) and bovine serum albumin as a standard. 30–40 μg of cell lysate were separated by discontinuous SDS-polyacrylamide gel electrophoresis and electrotransferred onto Hybond-C nitrocellulose membrane (Amersham Biosciences) using standard procedures. The resulting blot was blocked with 5% skim milk in phosphate-buffered saline containing 0.1% Tween 20 and next incubated with a designed primary antibody in phosphate-buffered saline containing 5% bovine serum albumin and 0.1% Tween 20 overnight at room temperature. Proteins were detected with a secondary antibody conjugated to horseradish peroxidase and an enhanced chemiluminescence commercial kit (Amersham Biosciences). Antibodies to p90^{Rsk} phosphorylated at

Ser^{380}; Akt phosphorylated at Ser^{473} or Thr^{308}; p70 S6 kinase phosphorylated at Thr^{389}; p44/42 mitogen-activated protein kinase phosphorylated at Thr^{202} and Tyr^{204}; GSK-3α/β phosphorylated at Ser^{21} and Ser^9, respectively; and GSK-3β phosphorylated at Ser^9 (Cell Signaling Technology, Beverly, MA) were used at 1:1000 dilution to detect the catalytically active forms of the kinases. Primary antibodies reactive to phospho-Bad Ser^{112}, phospho-Bad Ser^{155}, Akt, Bad, Bax, and IκBα (Cell Signaling Technology) were used at 1:1000 dilution. Anti-poly(ADP-ribose) polymerase antibody (BD PharMingen) was used at 1:4000 dilution. Anti-cytochrome *c* antibody (BIOSOURCE, International, Camarillo, CA) was used at 1 μg/ml. Anti-porin 31HL/VDAC antibody (Calbiochem) was used at 2 μg/ml. Antibodies against β-catenin (BD PharMingen) were used at 1:500 dilution, and antibodies against actin (Sigma) were used at 1:5000 dilution. The anti-actin and anti-IκBα polyclonal antibodies were utilized to monitor loading and transfer conditions. Densitometry was performed on blots exposed on Biomax MR film (Eastman Kodak Co.) using a Hewlett-Packard ScanJet 3p scanner and NIH Image software.

Transient Transfections—Transfection of BHK-rGLP-2R cells was done in 60-mm dishes using Lipofectin reagent (Invitrogen) according to the manufacturer's protocol. For the cell survival assay, cultures were transfected with 1 μg of a Rous sarcoma virus-β-galactosidase expression plasmid plus 3 μg of pBluescript II (Stratagene, La Jolla, CA) carrier DNA or with the β-galactosidase reporter plasmid plus 3 μg of MtR(AB) expression vector. After transfection, cultures were maintained in serum-depleted medium for 15–17 h and then incubated with LY294002 in the presence or absence of the indicated drugs. Adherent cells were collected, and β-galactosidase activity was determined as described previously (13). The loss of β-galactosidase activity in these assays reflects cell death and elimination of the transfected cells (14).

For assessment of the site-specific phosphorylation of Bad in response to different agonists, cells were transfected with 1.4 μg of the pEBG-mBad expression plasmid (Cell Signaling Technology) and carrier DNA for a total of 4 μg. Following transfection, cells were incubated for 24 h in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and for 18–20 h in serum-depleted medium. Cultures were treated with the indicated drugs and then prepared for immunoblot analysis as described above.

Mitochondrial and Cytosolic Isolation—Membrane fractions enriched in mitochondrial or cytosolic fractions were prepared by differential centrifugation as previously described (13). Protein concentration was determined before preparing the samples for immunoblot analysis as specified above.

Statistical Analysis—For assessment of statistical significance, data were analyzed using analysis of variance, and group comparisons were done using Bonferroni's multiple comparison post-test.

RESULTS

To ascertain the importance of PI3K-dependent signaling for GLP-2-mediated cell survival, we examined the effect of the PI3K inhibitor LY294002 on viability in BHK-rGLP-2R cells. Incubation with LY294002 alone induced a marked reduction in cell viability, whereas co-administration of GLP-2 in the presence of LY294002 for 6.5 h significantly increased cell survival (Fig. 1*A*). The inhibitory effect of GLP-2 on LY294002-induced cell death was mimicked by forskolin (Fig. 1*A*), suggesting that cAMP plays a role in the protective effect of GLP-2.

We have previously shown that cycloheximide at concentrations that inhibit translation by >95% induces cell death and the biochemical and morphological changes of apoptosis in BHK-rGLP-2R cells (13). The combined administration of both LY294002 and cycloheximide markedly reduced cell viability to ~12%, clearly below that observed with either agent alone (Fig. 1*A*). However, treatment of cells with either GLP-2 or forskolin significantly reduced cell death induced by these agents (Fig. 1*A*), indicating that *de novo* protein synthesis is not required for the inhibitory effect of GLP-2 or forskolin on LY294002-induced cell death.

To determine the time period required for coupling of GLP-2R activation to a reduction in LY294002-associated cell death, separate groups of cells were pretreated with LY294002, following which either GLP-2 or forskolin was added at various time points prior to analysis of cell viability 7.3 h after addition
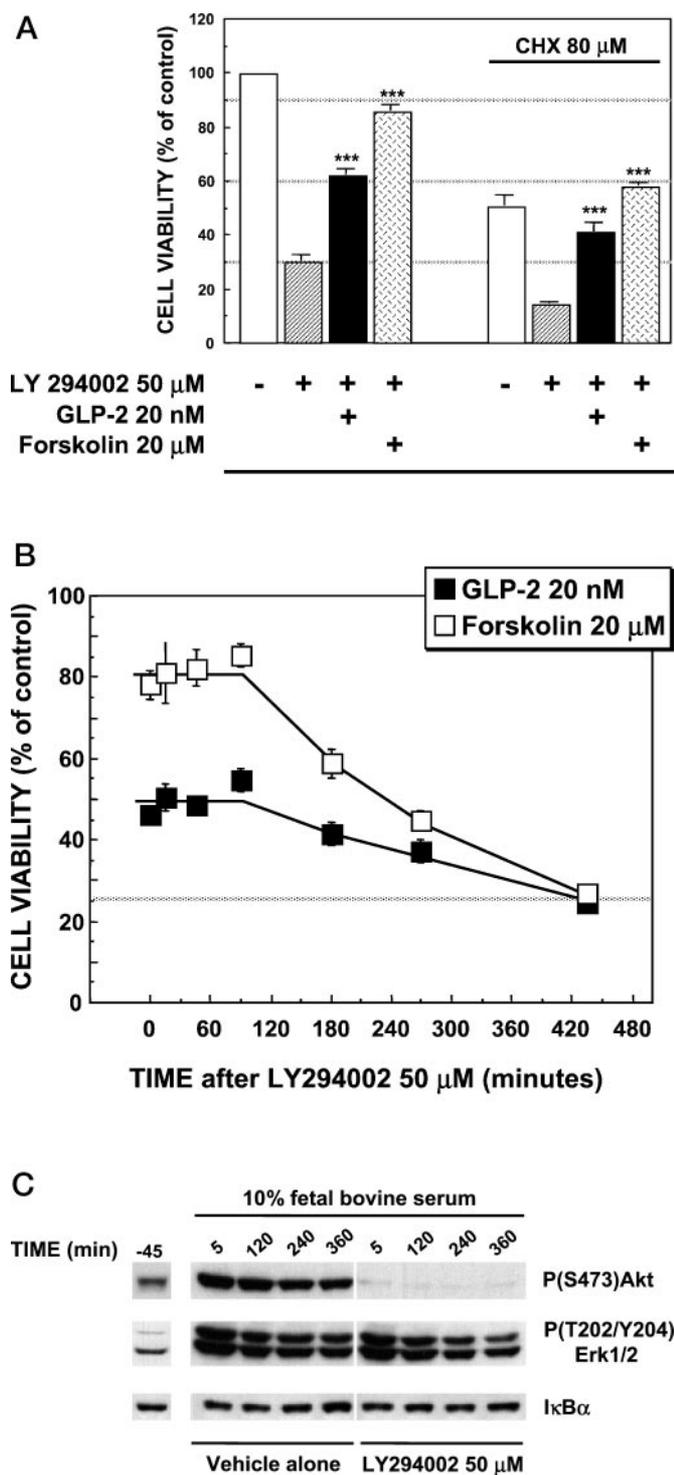
GLP-2R Activation Prevents Apoptosis

FIG. 1. **GLP-2 and forskolin attenuate cell death following inhibition of PI3K by LY294002 in BHK-rGLP-2R cells.** *A*, cells cultured as described under "Experimental Procedures" were pretreated with LY294002 alone or in combination with cycloheximide (*CHX*) for 45 min prior to adding GLP-2, forskolin, or vehicle for an additional 6.5 h. Cell viability was then assessed using a tetrazolium salt bioreduction assay and expressed as a percentage of the values obtained from analysis of vehicle-treated control cultures. Data are the means ± S.E. from seven (LY294002 alone) or four (LY294002 + cycloheximide pretreatment) independent experiments, each performed in quadruplicate. ***, $p < 0.001$ (LY294002 or LY294002 + cycloheximide + either GLP-2 or forskolin *versus* LY294002 or LY294002 + cycloheximide alone, respectively). *B*, GLP-2 or forskolin was added to the cultures at the indicated times following initiation of LY294002 treatment, and cell viability was determined as described for *A* after 7.3 h of exposure to LY294002. Data are the means ± S.D. of quadruplicate determinations from one representative experiment of two with similar

of LY294002 as shown in Fig. 1*B*. Both GLP-2 and forskolin significantly enhanced cell viability at all time points examined (Fig. 1*B*).

The effectiveness of LY294002 for sustained inhibition of PI3K in BHK-rGLP-2R cells during the 6–7-h time frame utilized for the viability experiments was assessed by examining the phosphorylation of the protein kinase Akt, a downstream target of PI3K signaling (15). As PI3K-dependent phosphorylation at Thr[308] and Ser[473] is necessary and sufficient for the activation of Akt, we used phosphorylation site-specific antibodies to detect catalytically active Akt as a marker of PI3K activation *in vitro*. Western blot analysis of serum-starved BHK-rGLP-2R cells re-treated with fetal bovine serum demonstrated a robust Akt Ser[473] phosphorylation from 5 to 360 min following exposure to serum (Fig. 1*C*). In contrast, co-administration of LY294002 completely abrogated the serum-stimulated phosphorylation of Akt Ser[473] during the entire time course of the experiment (Fig. 1*C*). These results illustrate the efficacy of LY294002 as an inhibitor of PI3K/Akt signaling in BHK-rGLP-2R cells. The relative specificity of LY294002 was confirmed by demonstrating preserved serum-stimulated ERK1/2 phosphorylation in BHK-rGLP-2R cells in the presence or absence of LY294002 (Fig. 1*C*).

Following treatment with LY294002, BHK-rGLP-2R cells exhibited morphological features typically associated with cell apoptosis, including membrane blebbing, cell shrinkage, and detachment as well as cell fragmentation into apoptotic bodies (Ref. 13 and data not shown). To ascertain whether reduced LY294002-associated cell viability is associated with biochemical hallmarks of apoptosis, we assessed mitochondrial release of apoptogenic cytochrome *c* and, separately, caspase activation by monitoring the cleavage of characteristic effector caspase substrates in the presence and absence of LY294002. Cells treated with LY294002 alone exhibited reduced levels of total Akt and cleavage of poly(ADP-ribose) polymerase and β-catenin (Fig. 2, *A* and *B*), and these effects were prevented by the pan-caspase inhibitor benzyloxycarbonyl-VAD-fluoromethyl ketone (Fig. 2*A*). In contrast, cells treated with LY294002 and either GLP-2 or forskolin exhibited preserved levels of Akt and reduced cleavage of poly(ADP-ribose) polymerase and β-catenin (Fig. 2*B*). LY294002 treatment of BHK-rGLP-2R cells was also associated with an increase in the levels of cytosolic cytochrome *c* that was reduced by GLP-2 or forskolin (Fig. 2*C*). Taken together, these experiments demonstrate that GLP-2 enhances cell survival and reduces mitochondrial cytochrome *c* efflux, caspase activation, and the subsequent cleavage of downstream targets of executioner caspases in a PI3K- and Akt-independent manner.

Engagement of the GLP-1 and GLP-2 receptors leads to increased adenylate cyclase activity, increased levels of intracellular cAMP, and activation of protein kinase A (PKA) (1). In the setting of cycloheximide-induced apoptosis, GLP-2 reduces caspase activation and enhances cell survival in a PKA-independent manner (13). Surprisingly, the LY294002-mediated reduction in BHK-rGLP-2R cell viability was not reversed by GLP-2 or forskolin in the presence of the PKA inhibitor H89 (Fig. 3). A transient transfection death assay was used to verify

results. *C*, LY294002 produced sustained inhibition of Akt activation in BHK-rGLP-2R cells. Cells were serum-deprived for 17 h and then stimulated with 10% fetal bovine serum for the indicated periods of time following a 45-min preincubation with LY294002 or vehicle alone. Cell extracts were then analyzed by immunoblotting for phosphorylated Akt (*P(S473)Akt*) and phosphorylated ERK1/2 (*P(T207/Y204)Erk1/2*) as described under "Experimental Procedures." Anti-IκBα antibody was used to monitor loading and transfer conditions. Results are representative of four independent experiments.

FIG. 2. **GLP-2 and forskolin prevent both LY294002-induced caspase activation and mitochondrial cytochrome *c* release in BHK-rGLP-2R cells.** *A*, caspase-dependent cleavage of Akt and β-catenin following treatment with LY294002. Cells were pretreated with 65 μM benzyloxycarbonyl-VAD-fluoromethyl ketone (*z-VAD-fmk*) for 30 min prior to incubation with LY294002 or vehicle alone for 5 h. Total cell extracts were then analyzed by immunoblotting for Akt and β-catenin. Equal loading was monitored by probing the blots for IκBα. Results are representative of two independent experiments. *B*, effect of GLP-2 and forskolin on LY294002-induced cleavage of effector caspase substrates. Cells were exposed to GLP-2 or forskolin (*Fk*) for 6.5 h following a 45-min preincubation with LY294002 or vehicle alone (*cont*). Western blot analysis was performed to detect Akt, poly(ADP-ribose) polymerase (*PARP*), and β-catenin in the cell extracts as shown in the *left panels*. Equal loading was verified by reprobing the blots with anti-actin antibody. The intensity of the Akt, intact poly(ADP-ribose) polymerase and intact β-catenin signals was quantified by densitometry; corrected by the intensity of the actin signal; and expressed relative to the values for vehicle-treated control cultures in the *right panels*. Data are the means ± S.D. from four (Akt) or three (poly(ADP-ribose) polymerase and β-catenin) independent experiments. *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001 (LY294002 + either GLP-2 or forskolin *versus* LY294002 alone). *C*, mitochondrial cytochrome *c* efflux in cells exposed to LY294002 in the presence or absence of GLP-2 or forskolin for 5 h. Cytosolic supernatants were prepared, and Western blot analysis was performed to detect cytochrome *c* (*cyt c*). The quality of the subcellular fractionation and equivalent protein loading per lane were monitored by probing the blots for porin and IκBα, respectively. A mitochondrial fraction sample (*Mit*) from untreated cells was used as a positive control for the presence of both cytochrome *c* and porin. Results are representative of three independent experiments.
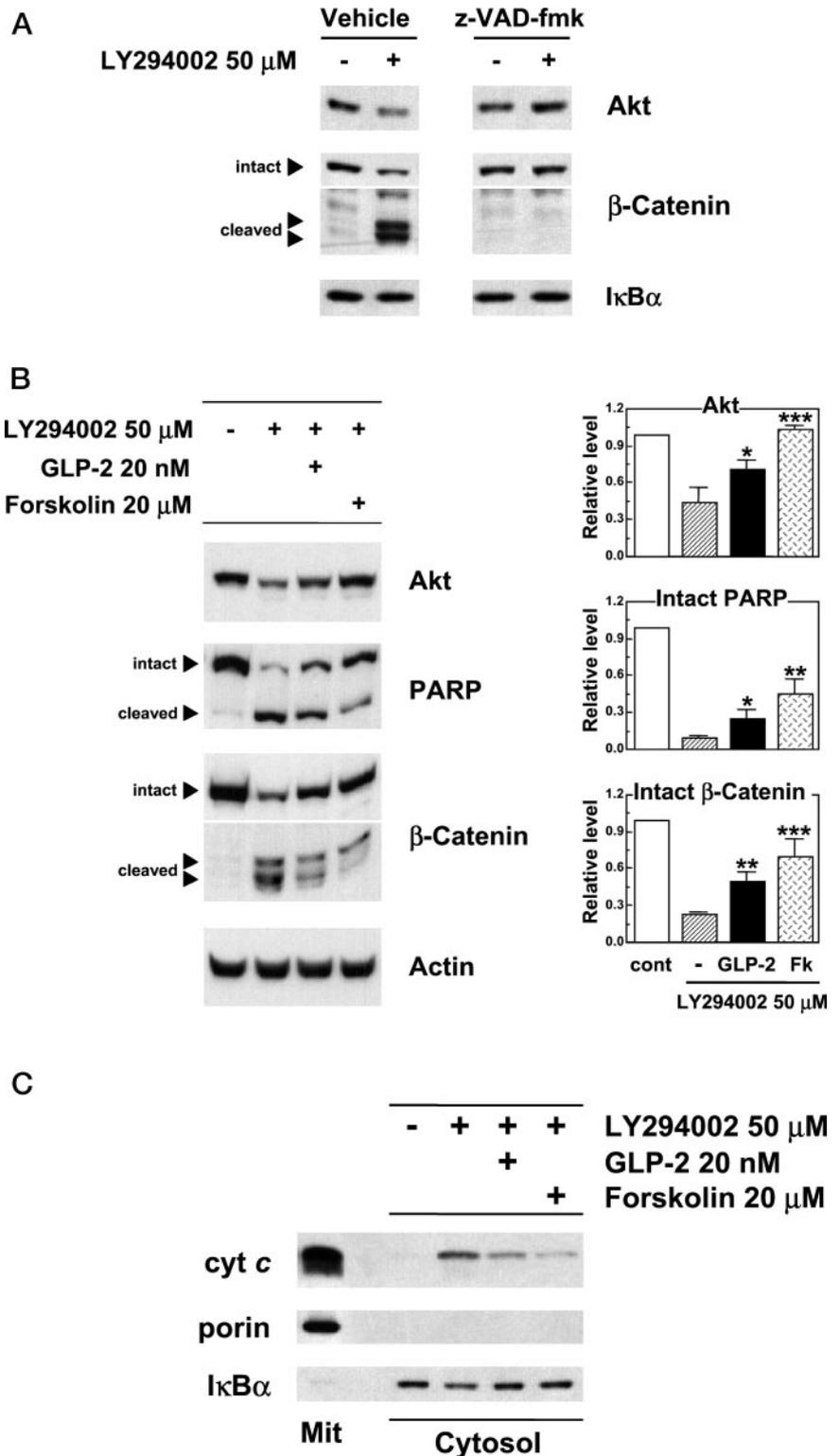
the PKA dependence of the GLP-2 effect on cell survival in the presence of LY294002. Cells were cotransfected with the Rous sarcoma virus-β-galactosidase reporter plasmid alone or in combination with MtR(AB), a vector encoding a dominant-negative regulatory subunit of PKA (16), and then exposed to LY294002 with or without GLP-2 or forskolin. The amount of β-galactosidase activity detected in intact adherent cells provides a readout for the number of viable transfected cells, whereas a decrease in reporter gene activity indicates cell death (14). In contrast to the levels of β-galactosidase activity

detected in GLP-2-treated cells co-incubated with LY294002, cotransfection with MtR(AB) completely eliminated the GLP-2- or forskolin-stimulated enhancement of β-galactosidase activity in BHK-rGLP-2R cells exposed to LY294002 (Fig. 3), providing complementary evidence that PKA activity is required for the protective effect of GLP-2 and forskolin on LY294002-induced cell death.

These results implied that the GLP-2- and forskolin-mediated rescue of BHK-rGLP-2R cells from LY294002-induced apoptosis required PKA activity, but was likely independent of
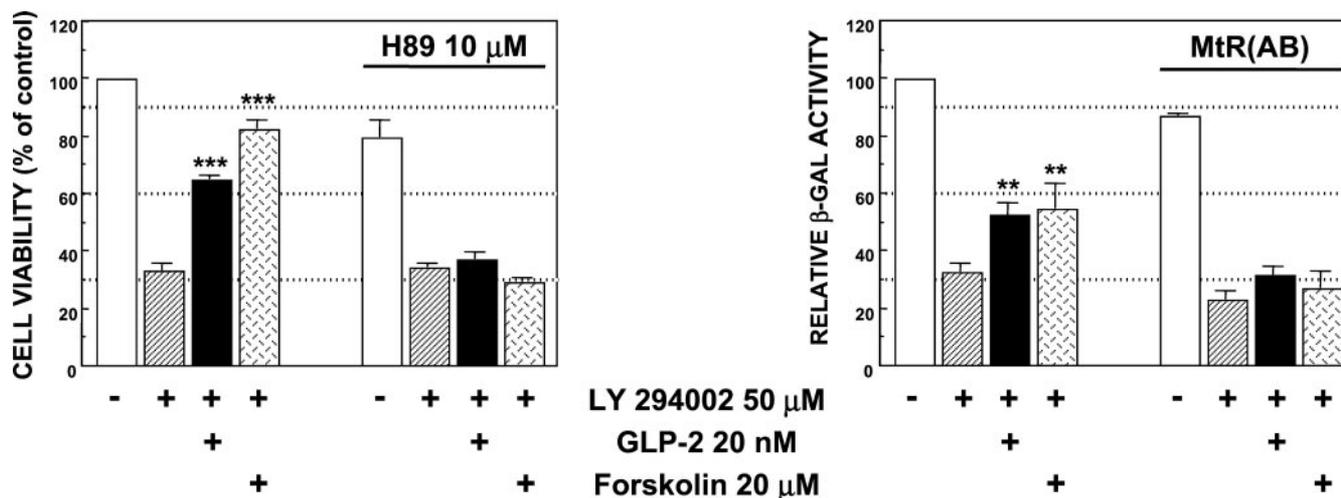
FIG. 3. **GLP-2 and forskolin protect BHK-rGLP-2R cells from LY294002-induced apoptosis in a PKA-dependent manner.** *Left panel*, cultures were pretreated with LY294002 alone or in combination with H89 for 45 min prior to adding GLP-2 or forskolin. After 6.5 h, cell viability was determined as described for Fig. 1*A* and expressed as a percentage of the values for vehicle alone-treated control cells. Data are the means ± S.E. from three independent experiments, each performed in quadruplicate. ***, $p < 0.001$ (LY294002 + either GLP-2 or forskolin *versus* LY294002 alone). *Right panel*, cells were transfected with Rous sarcoma virus-β-galactosidase alone or in combination with the dominant-negative PKA expression plasmid MtR(AB). After 16 h, cultures were treated for 45 min with LY294002 and then with GLP-2 or forskolin for 8 h. β-Galactosidase (*β-GAL*) activity was determined in cell lysates and expressed as a percentage of the activity in vehicle-treated control cultures. Data are the means ± S.E. from two independent transfections, each performed in triplicate. **, $p < 0.01$ (LY294002 + either GLP-2 or forskolin *versus* LY294002 alone).

Akt activation, as LY294002 is a potent and long-lasting inhibitor of PI3K and Akt activation under the experimental conditions used for our studies (see Fig. 1*C*). However, cAMP and PKA have been shown to induce a PI3K-independent activation of Akt that is dependent on Thr[308] phosphorylation, but not Ser[473] phosphorylation (17, 18), raising the possibility that PKA might transduce GLP-2-mediated survival in BHK-rGLP-2R cells via regulation of Akt. To address whether GLP-2 activates Akt in BHK-rGLP-2R cells, we incubated cells with GLP-2 or forskolin for 5–60 min, following which the phosphorylation state of Akt was assessed by Western blotting using phosphorylation-specific antisera directed against Ser[473] and Thr[308]. Whereas insulin treatment of BHK-rGLP-2R cells rapidly induced Akt phosphorylation within 15 min, no phosphorylation of Akt at the two critical residues that activate the kinase was detected following treatment with forskolin or GLP-2 (Fig. 4). When the same experiments were performed in the presence of LY294002, the levels of phosphorylated Akt were markedly reduced, and no evidence for either GLP-2- or forskolin-induced Akt phosphorylation was observed (data not shown). These observations suggest that Akt does not mediate the survival effects of cAMP/PKA in LY294002-treated BHK-rGLP-2R cells.

As p70 S6 kinase is a downstream effector of PI3K and may potentially regulate changes in cell proliferation or apoptosis (19), we examined whether GLP-2 activates p70 S6 kinase in the presence or absence of LY294002 using anti-phospho-p70 S6 kinase Thr[389] antibody, as the activity of p70 S6 kinase *in vivo* most closely correlates with the phosphorylation state of Thr[389] (20). Although insulin activated Thr[389] phosphorylation of p70 S6 kinase in BHK-rGLP-2R cells, no increase in Thr[389] phosphorylation was detected following exposure of cells to forskolin or GLP-2 (Fig. 5). Furthermore, LY294002 completely abolished p70 S6 kinase Thr[389] phosphorylation in the presence or absence of GLP-2 or forskolin (data not shown). Similarly, we next examined whether GLP-2 might exert its effects on cell survival via activation of p90[Rsk], a ribosomal kinase that exerts anti-apoptotic effects on downstream effectors such as Bad (21, 22). Although phorbol esters stimulated phosphorylation of p90[Rsk] at Ser[380], which is critical for the activation of

this kinase (23), GLP-2 or forskolin had no effect on p90[Rsk] Ser[380] phosphorylation in the presence (data not shown) or absence (Fig. 5) of LY294002.

To determine the mechanisms underlying the reduced cell survival in LY294002-treated BHK-rGLP-2R cells and to identify candidate GLP-2-responsive molecules that control cell survival, we examined the phosphorylation state of GSK-3, a downstream target of both the PI3K/Akt (24) and cAMP/PKA (25, 26) anti-apoptotic signaling pathways and whose activity suppresses proliferation and induces cell death (24). In mammalian cells, activation of Akt and PKA in response to a variety of physiological stimuli has been demonstrated to rapidly phosphorylate GSK-3 at Ser[21] in GSK-3α and at Ser[9] in GSK-3β, resulting in inhibition of GSK-3 kinase activity and promotion of survival (24–26).

Incubation of BHK-rGLP-2R cells with LY294002 produced a rapid reduction in the levels of Akt Ser[473] phosphorylation, which was mirrored by a comparably rapid reduction in the levels of catalytically inactive GSK-3α and GSK-3β phosphorylated at Ser[21] and Ser[9], respectively (Fig. 6). Stimulation of BHK-rGLP-2R cells with GLP-2 and, to a greater extent, with forskolin increased the levels of phosphorylated GSK-3α and GSK-3β at Ser[21] and Ser[9], respectively (Fig. 7, *A* and *C*). The ability of GLP-2 and forskolin to augment levels of catalytically inactive phosphorylated GSK-3 was reversed by H89 (Fig. 7, *B* and *C*), implicating an essential role for PKA as a downstream effector connecting GLP-2R signaling to GSK-3 phosphorylation independently of PI3K/Akt, p90[Rsk], and p70 S6 kinase.

The available data suggested that GLP-2 might enhance cell survival in the presence of LY294002 via GSK-3 inactivation, thereby preventing GSK-3-dependent apoptotic cell death. Indeed, inhibition of GSK-3, either pharmacologically or genetically by transfection with kinase-inactive GSK-3 alleles, has been shown to prevent apoptosis induced by inhibition of PI3K (27–29). Consistent with this hypothesis, the GSK-3 inhibitor LiCl significantly increased cell survival in LY294002-treated BHK-rGLP-2R cells, and GLP-2 treatment further increased cell viability in either the presence or absence of LiCl (Fig. 8). These findings provide additional evidence correlating GSK-3 activity with survival of BHK-rGLP-2R cells and suggest that
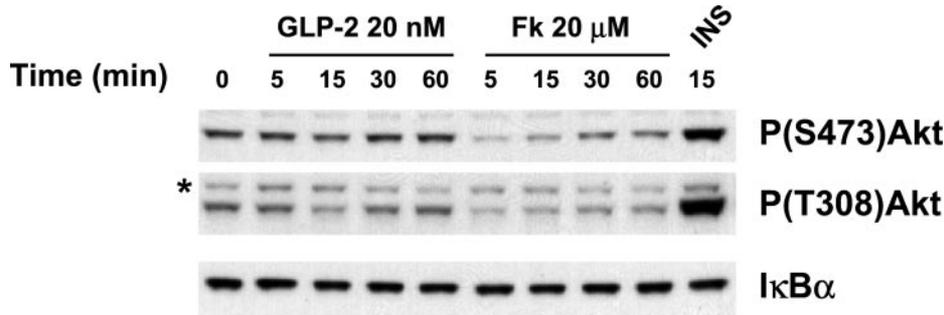
FIG. 4. **GLP-2 and forskolin do not mediate Akt phosphorylation in BHK-rGLP-2R cells.** Serum-starved cultures were treated for the indicated times with GLP-2, forskolin (*Fk*), or 100 nM insulin (*INS*). Levels of catalytically active Akt (phosphorylated at Ser[473] and Thr[308] (*P(S473)Akt* and *P(T308)Akt*, respectively)) in the corresponding cell extracts were measured by Western blot analysis using Akt phosphorylation site-specific antibodies. Anti-IκBα antibody was used to monitor loading and transfer conditions. The *asterisk* indicates the position of a nonspecific band cross-reacting with the anti-phospho-Akt Thr[308] antibody. Results are representative of four independent experiments.
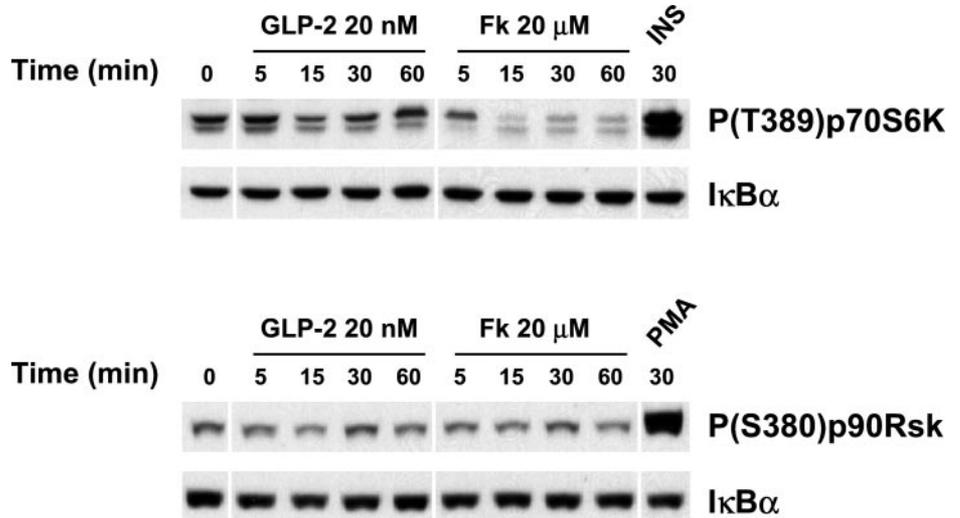
FIG. 5. **GLP-2 or forskolin does not activate p70 S6 kinase or p90[Rsk] in BHK-rGLP-2R cells.** Cells were treated for the indicated times with GLP-2, forskolin (*Fk*), 100 nM insulin (*INS*; positive control for p70 S6 kinase activation), or 400 nM PMA (positive control for p90[Rsk] activation). Cell lysates were immunoblotted with anti-phospho-p70 S6 kinase Thr[389] (*P(T389)p70S6K*), anti-phospho-p90[Rsk] Ser[380] (*P(S380)p90Rsk*), or anti-IκBα (loading control) antibody. Results are representative of three independent experiments.
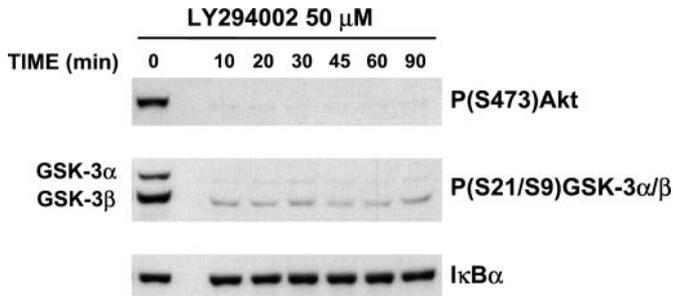

FIG. 6. **LY294002 treatment rapidly inhibits basal Akt and GSK-3 phosphorylation in BHK-rGLP-2R cells.** Cultures were serum-deprived for 16 h and then treated with LY294002 for the indicated times. Total cell extracts were analyzed by immunoblotting for catalytically active phosphorylated Akt and for catalytically inactive phosphorylated GSK-3 using anti-phospho-Akt Ser[473] (*P(S473)Akt*) and anti-phospho-GSK-3α/β Ser[21/9] (*P(S21/S9)GSK-3α/β*) antibodies, respectively. Equal loading was verified by probing the blots with anti-IκBα antibody. Results are representative of three independent experiments.

the prosurvival actions of GLP-2 are not completely identical to those mediated by the effects of LiCl on GSK-3.

Survival factors acting through kinases such as Akt, PKA, p90[Rsk], and p70 S6 kinase induce phosphorylation of the proapoptotic Bcl-2 protein Bad, which promotes survival by preventing its interaction with mitochondrially associated prosurvival Bcl-2 family members and induces sequestration of Bad away from the mitochondria following binding of 14-3-3 proteins (30, 31). As we were unable to detect endogenous levels of phosphorylated Bad in BHK-rGLP-2R cell extracts, we examined site-specific Bad phosphorylation in cells transiently

transfected with an expression vector encoding a glutathione *S*-transferase-Bad fusion protein.

Although both Bad Ser[112] and Ser[155] have been shown to be phosphorylated by PKA *in vivo* (32–35), treatment of BHK-rGLP-2R cells with forskolin, which stimulates PKA through the activation of adenylyl cyclase, specifically induced enhanced Bad phosphorylation at Ser[155] without affecting Bad Ser[112] phosphorylation (Fig. 9). Akt has also been reported to be a Bad Ser[155] kinase (33); however, serum treatment of the cells activated Akt without concomitant change in Bad Ser[155] phosphorylation, yet increased, as did phorbol ester, the levels of phosphorylated Bad at Ser[112] (Fig. 9). Both serum and PMA also strongly activated p90[Rsk] (Fig. 9), which can act as a Bad Ser[112] kinase (21, 22). These results suggest that PKA (but not Akt) is primarily a Bad Ser[155] kinase in BHK-rGLP-2R cells.

To ascertain whether the prosurvival effects of GLP-2 and forskolin detected following inhibition of PI3K are related to Bad phosphorylation, the levels of Bad phosphorylated at Ser[155] were examined in cells treated with LY294002. The results of these experiments demonstrated that both GLP-2 and forskolin augmented the levels of Bad phosphorylated at Ser[155] independently of the presence or absence of LY294002 (Fig. 10*A*). As the GLP-2-mediated enhancement of cell survival following exposure to LY294002 was PKA-dependent, we assessed the extent of Bad Ser[155] phosphorylation in BHK-rGLP-2R cells incubated with or without the PKA inhibitor H89. Western blot analysis demonstrated that the GLP-2 stimulation of Bad Ser[155] phosphorylation was completely abrogated in BHK-rGLP-2R cells treated concomitantly with H89 (Fig. 10*B*).
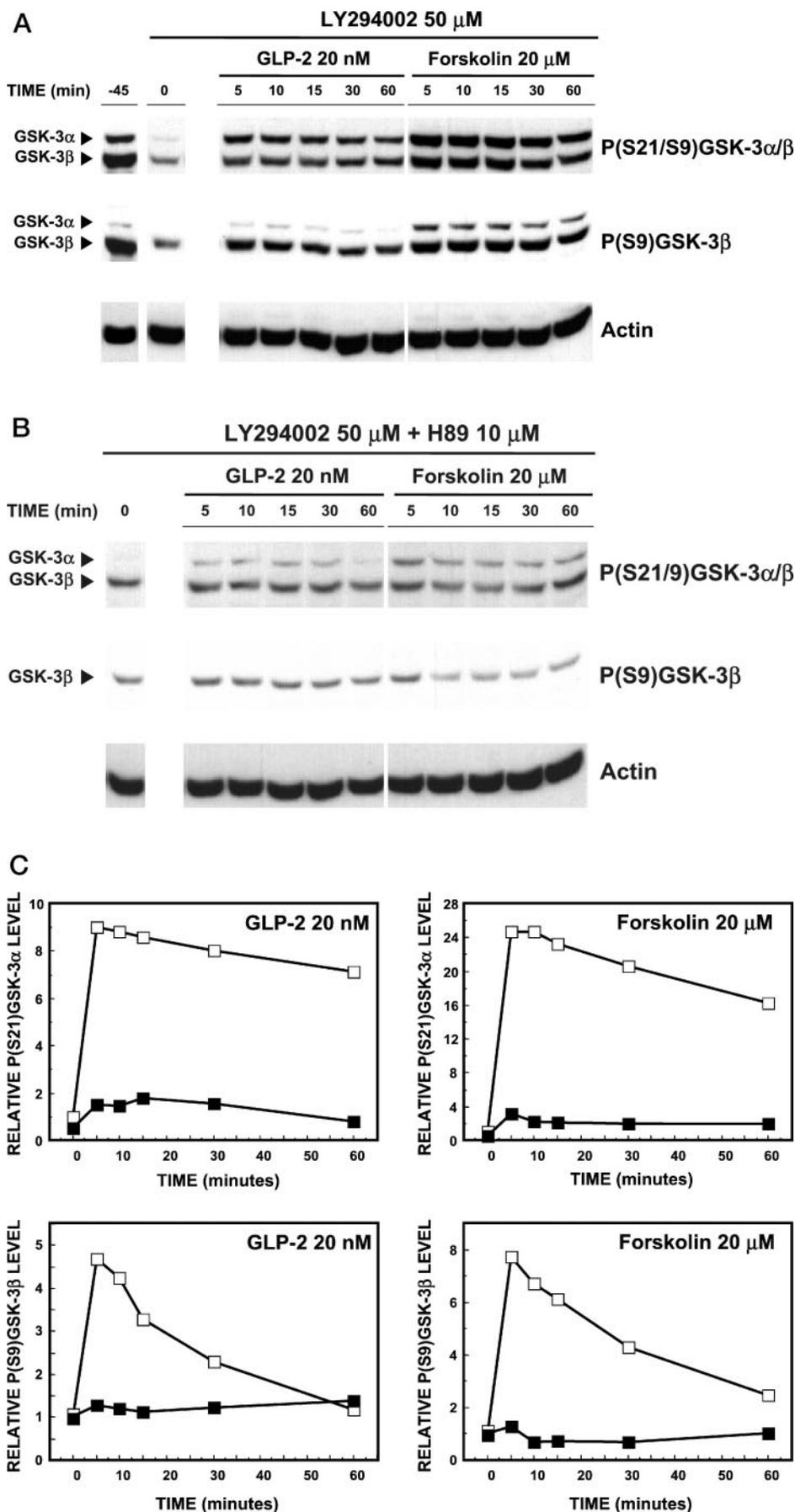
FIG. 7. **GLP-2 and forskolin induce phosphorylation of GSK-3 in a PI3K-independent, PKA-dependent manner in BHK-rGLP-2R cells.** Serum-starved cultures were incubated for 45 min with LY294002 (*A*) or LY294002 + H89 (*B*) before stimulation by GLP-2 and forskolin for the indicated times. Extracts were prepared, and Western blot analysis was performed to monitor GSK-3 phosphorylation. Anti-phospho-GSK-3α/β Ser[21/9] (*P(S21/S9)GSK-3α/β*) antibody was used to detect GSK-3α phosphorylation at Ser[21]. GSK-3β phosphorylation was examined using anti-phospho-GSK-3β Ser[9] (*P(S9)GSK-3β*) antibody, which demonstrated higher sensitivity than anti-phospho-GSK-3α/β Ser[21/9] antibody for the β isoform of GSK-3. Loading and transfer conditions were verified by reprobing the blots with anti-actin polyclonal antibody. Shown in *C* are the levels of catalytically inactive phosphorylated GSK-3α and GSK-3β from the experiments illustrated in *A* (□) and *B* (■), determined by densitometry as described under "Experimental Procedures" and normalized to the corresponding phosphorylation levels at time 0. Results are representative of four independent experiments.
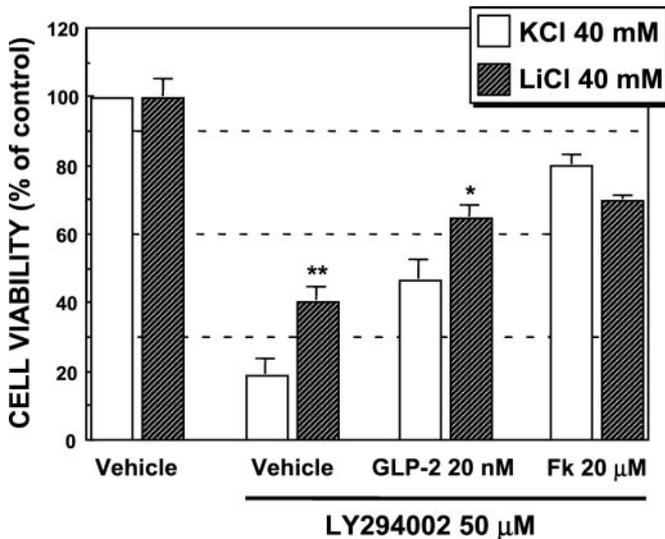
FIG. 8. **Effect of lithium treatment on LY294002-induced cell death.** Cultures were treated with 40 mM lithium chloride or potassium chloride (to monitor any effect of the osmolarity change on cell viability). After 1 h, LY294002 was added to the cultures, followed by GLP-2 and forskolin (*Fk*) 45 min later. Cell viability was determined after a further 6.5-h incubation and expressed as a percentage of the values from cultures exposed to KCl alone. Data are the means ± S.E. from three independent experiments, each performed in quadruplicate. **, $p < 0.01$ (LY294002 + LiCl *versus* LY294002 + KCl); *, $p < 0.05$ (LY294002 + LiCl + GLP-2 *versus* LY294002 + KCl + GLP-2).
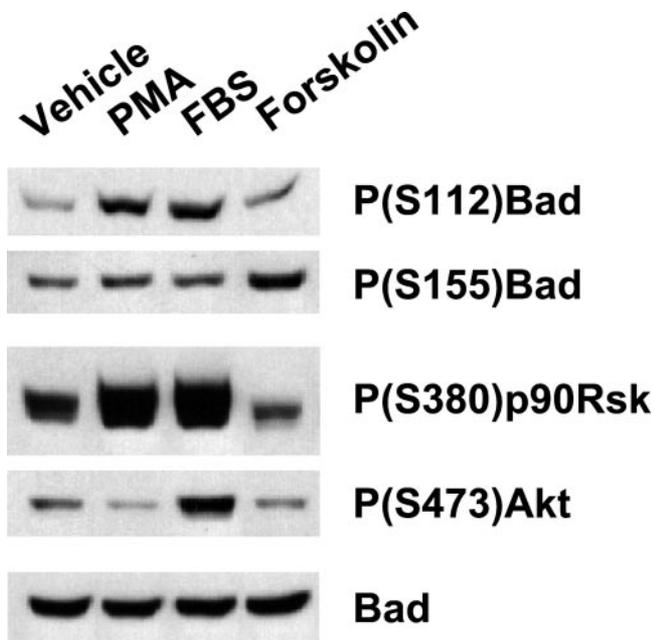

FIG. 9. **Forskolin induces phosphorylation of Bad at Ser[155] (but not at Ser[112]) in BHK-rGLP-2R cells.** Cultures were transfected with a plasmid encoding a glutathione *S*-transferase-Bad fusion protein. After 24 h, cells were serum-deprived; and 18 h later, they were treated with 400 nM PMA, 10% fetal bovine serum (*FBS*), or 20 μM forskolin for 20 min. Total cell extracts were then analyzed by immunoblotting for phosphorylated Bad (*P(S112)Bad* and *P(S155)Bad*), phospho-p90[Rsk] Ser[380] (*P(S380)p90Rsk*), and phospho-Akt Ser[473] (*P(S473)Akt*) using phosphorylation site-specific antibodies. Equal loading was verified by probing the blots with a phosphorylation-independent Bad antibody. Results are representative of two independent experiments.

Phosphorylation of Bad specifically at Ser[155] has been shown to disrupt the binding of Bad to prosurvival Bcl-2 family proteins, thus inducing translocation of Bad from the outer mitochondrial membrane to the cytoplasm (33). If phosphorylation

of Bad at Ser[155] contributes to the prosurvival effects of GLP-2 and forskolin following LY294002-induced apoptosis, it should lead to sequestration of endogenous Bad away from the mitochondria. Whereas LY294002 treatment promoted a 2–3-fold increase in the level of Bad bound to the mitochondrial heavy membrane fraction, both GLP-2 and forskolin significantly reduced mitochondrially associated Bad (Fig. 11). In response to apoptotic signals, Bax, a pro-apoptotic Bcl-2 family member that is normally located in the cytosol, translocates to the mitochondria, where it triggers rapid cytochrome *c* release (36, 37). Following exposure of cells to LY294002, the levels of mitochondrially associated Bax mirrored those of Bad in the presence or absence of GLP-2 or forskolin (Fig. 11) and correlated with the appearance of cytochrome *c* in the cytosol (see Fig. 2*C*). Taken together, these results suggest that GLP-2 induces dissociation of the pro-apoptotic Bcl-2 family members Bad and Bax from the mitochondria, contributing to the cytoprotective actions of GLP-2 following inhibition of PI3K/Akt signaling in BHK-rGLP-2R cells.

DISCUSSION

The observation that GLP-2 attenuates cellular injury *in vivo* has fostered efforts directed at understanding how activation of the GLP-2R activates a prosurvival signal either indirectly in the intestinal mucosa or directly in cells expressing the GLP-2R. The human GLP-2R has been localized to subsets of enteroendocrine cells in the small and large bowel epithelia using receptor-specific antisera (38). In contrast, the murine GLP-2R is not detected in intestinal endocrine cells, but GLP-2R RNA transcripts have been localized to enteric neurons by *in situ* hybridization (39). As enriched populations of enteroendocrine cells or enteric neurons that express an endogenous GLP-2R have not yet been isolated for detailed studies *in vitro*, we examined the direct effects of GLP-2R signaling in BHK fibroblasts stably transfected with the GLP-2R. We focused our current studies on the potential importance of the PI3K pathway in the control of GLP-2-regulated apoptosis, as the PI3K/Akt pathway has been shown to be critical for neuronal survival in several experimental systems (30).

Both GLP-1 and GLP-2 activate adenylyl cyclase in target cells and are presumed to signal predominantly through a cAMP-dependent pathway (1). As increased levels of cAMP commonly enhance cell survival, often through a PKA- and Akt-dependent pathway (17, 30), we initially hypothesized that GLP-2 would be unable to reverse LY294002-induced cell death due to the central importance of PI3K and Akt for control of cell survival. Remarkably, both GLP-2 and forskolin attenuated cell death even in the presence of LY294002, despite the complete absence of Akt activation. These findings are consistent with data demonstrating that, although increased levels of cAMP activate Akt independently of PI3K in 293 cells (40), Akt is not required for the prosurvival effects of cAMP, as cAMP analogs suppress caspase activation and reduce tumor necrosis factor α-mediated apoptosis in hepatocytes independently of Akt activation (40).

Our previous studies demonstrated that both GLP-2 and forskolin exhibit PKA-independent anti-apoptotic effects in cycloheximide-treated BHK-rGLP-2R cells (13). Although PKA activation is commonly required for anti-apoptotic action following G protein-coupled receptor (GPCR) activation, cAMP-dependent, yet PKA-independent effects on cell survival have been noted previously in studies of neutrophil apoptosis (41). Similarly, although cAMP agonists suppress tumor necrosis factor-mediated apoptosis in hepatocytes, this effect is only partially dependent on PKA activation (40). Furthermore, GLP-1 receptor activation has also been shown to exert downstream effects through cAMP-dependent, yet PKA-independent

A

FIG. 10. **GLP-2 and forskolin induce phosphorylation of Bad at Ser^{155} in a PKA-dependent, but PI3K-independent manner in BHK-rGLP-2R cells.** Cells were transfected with the pEBG-mBad expression plasmid, serum-deprived for 20 h, and then pretreated for 45 min with vehicle alone and either LY294002 (*A*) or H89 (*B*) before stimulation by GLP-2 and forskolin (*Fk*) for the indicated times. Cell extracts were prepared, and Western blot analysis was performed to detect Bad phosphorylated at Ser^{155} (*P(S155)Bad*) using anti-phospho-Bad Ser^{155} antibody. Loading and transfer conditions were monitored by reprobing the blots with a phosphorylation-independent Bad antibody. Illustrated at the bottom of *B* is the densitometric profile of the corresponding phospho-Bad Ser^{155} immunoblot. Results are representative of three independent experiments.
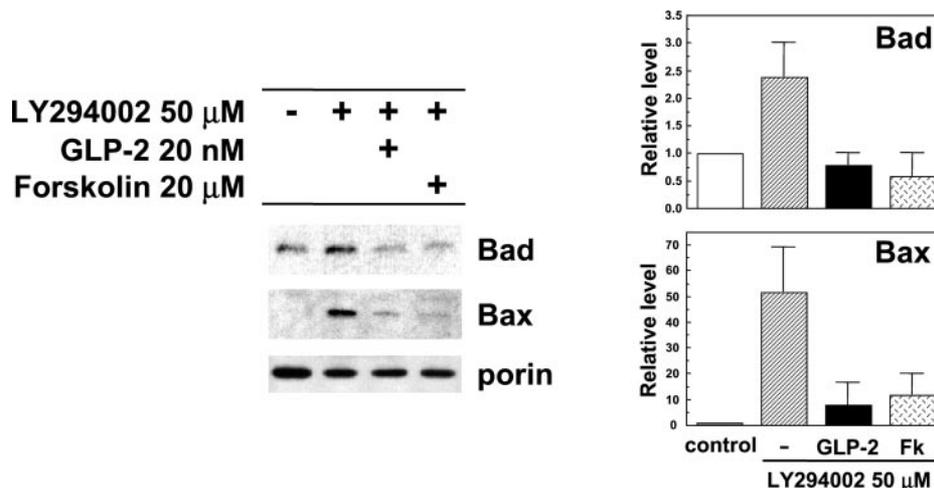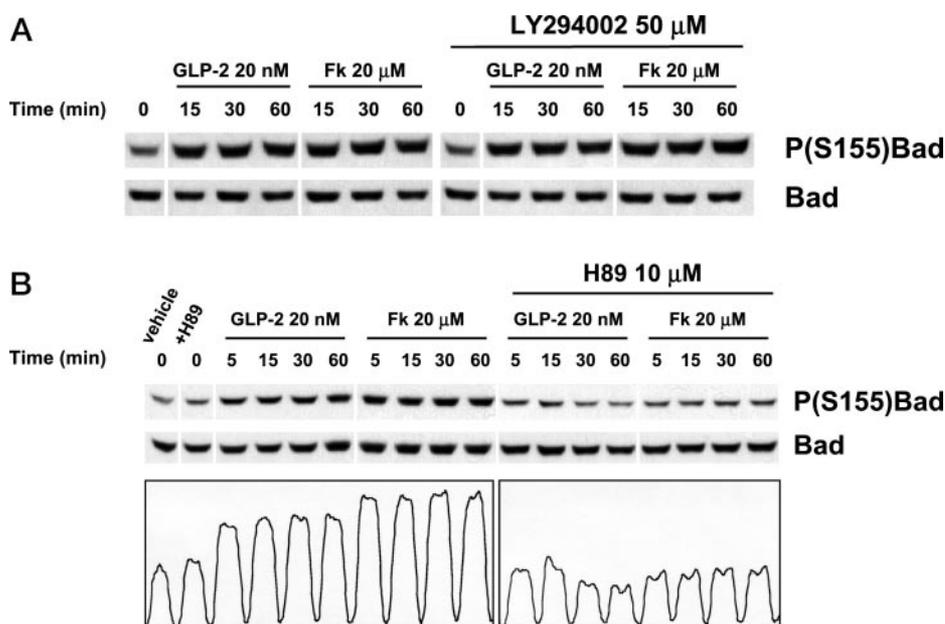
FIG. 11. **GLP-2 and forskolin attenuate LY294002-induced increases in pro-apoptotic Bad and Bax associated with mitochondria in BHK-rGLP-2R cells.** Cultures were exposed to GLP-2 or forskolin (*Fk*) for 6 h following a 45-min preincubation with LY294002 or vehicle alone. Mitochondrially enriched membrane pellets were then prepared, and Western blot analysis was performed to detect endogenous Bad and Bax as shown in the *left panels*. Equal loading was verified by reprobing the blots with anti-porin antibody. The intensity of the Bax and Bad signals in the mitochondrial fraction was quantified by densitometry, corrected by the intensity of the porin signal detected on the same blot, and expressed relative to the values for vehicle alone-treated control cultures in the *right panels*. Data are the means ± S.D. from three independent experiments.

actions in islet cells, likely via activation of cAMP-regulated guanine nucleotide exchange factors (42, 43). Hence, although PKA is clearly important for LY294002-dependent cell survival in BHK-rGLP-2R cells, PKA is likely to mediate many (but not all) of the events observed following activation of the GLP-2R cAMP-dependent signaling pathway in different cell systems.

PKA and Akt share several common downstream targets important for control of cell death, including cAMP-responsive element-binding protein, Bad, and GSK-3 (44). Both GLP-2 and forskolin enhanced Bad phosphorylation at Ser^{155} in BHK-rGLP-2R cells in a PI3K-independent manner, suggesting that GLP-2-stimulated enhancement of cell survival may be mediated in part by interaction of Bad with 14-3-3 proteins and the release of anti-apoptotic members of the Bcl-2 family such as Bcl-x$_L$ (33, 34). Although Rsk and p70 S6 kinase have been implicated as regulators of Bad phosphorylation (19, 34, 35), we did not detect evidence for GLP-2- or forskolin-stimulated phosphorylation of these kinases in BHK-rGLP-2R cells. In contrast, the GLP-2-stimulated phosphorylation of Bad at Ser^{155} was clearly PKA-dependent, consistent with the previously described importance of PKA for regulation of Bad activity at this specific serine residue (34, 35).

The GLP-2-mediated enhancement of cell survival was also associated with phosphorylation of both GSK-3α and GSK-3β in a PI3K-independent, PKA-dependent manner. Inhibition of GSK-3 activity via phosphorylation at Ser^{21} in GSK-3α and at Ser^9 in GSK-3β may occur via p90^{Rsk}, p70 S6 kinase, Akt, or PKA and is generally associated with reduction of apoptosis in both fibroblasts and neurons (24–26). Our finding that GLP-2 phosphorylated GSK-3 in BHK-rGLP-2R cells, taken together with the enhancement of cell survival detected following incubation of the cells with lithium chloride, clearly implicates GSK-3 as a downstream target for the direct actions of GLP-2R signaling on cellular apoptosis.

Activation of growth factor receptor signaling coupled to kinase cascades represents an established paradigm for controlling cell growth and apoptosis; however, increasing evidence suggests that activation of GPCRs also converges on pathways that regulate cell survival (Fig. 12). Parathyroid hormone-related hormone, bradykinin, corticotropin-releasing
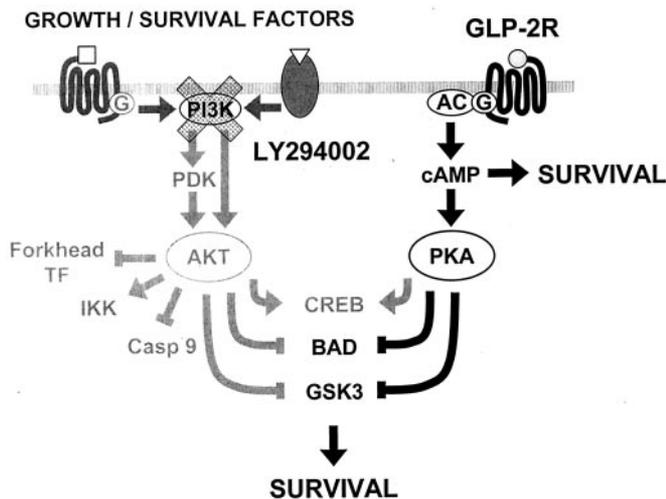
FIG. 12. **GLP-2 regulates LY294002-associated cell survival in a PKA-dependent manner via Bad and GSK-3.** Shown is a schematic representation of how GLP-2R activation signals (*thick lines*) to increase cell survival in BHK-rGLP-2R cells. *PDK*, phosphoinositide-dependent kinase; *TF*, transcription factor; *IKK*, IκB kinase; *Casp 9*, caspase-9; *CREB*, cAMP-responsive element-binding protein.

hormone, vasoactive intestinal peptide, opiates, pituitary adenylate cyclase-activating polypeptide, endothelins, adenosine, somatostatin, and lysophosphatidic acid all modulate cell death through GPCRs in a diverse number of cell types (45–52). Although activation of PKA is essential for prevention of cell death in some cell types, regulators of G protein signaling may also modulate GPCR-regulated apoptosis (53) independently of PKA (46).

GPCR activation leads to diverse and often opposing effects on cell survival via increasingly complex signaling mechanisms, even among highly related members of the same receptor superfamily. For example, β-adrenergic receptor agonists or cAMP analogs induce apoptosis in murine S49 lymphoma cells via $G\alpha_s$-dependent pathways (54, 55). Conversely, activation of GPCR signaling may attenuate apoptosis in activated T lymphocytes via suppression of Fas ligand, as both vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide down-regulate Fas ligand transcription through the type 2 vasoactive intestinal peptide receptor in a cAMP-dependent manner (56). Pituitary adenylate cyclase-activating polypeptide also exerts anti-apoptotic actions in cerebellar neurons via cAMP in a PKA-dependent manner (57).

In contrast, activation of cardiac β-adrenergic receptor signaling induces apoptosis in rat cardiac myocytes in a PKA-dependent manner (58, 59), whereas activation of α-adrenergic receptors antagonizes the apoptotic actions of 8-bromo-cAMP in the same cells (60). Furthermore, although activation of either β_1- or β_2-adrenergic receptors increases contractility via G_s-dependent coupling to adenylate cyclase, activation of β_2-adrenergic receptors inhibits apoptosis via a G_i-coupled pathway (61). Additional evidence for GPCR cross-talk in the control of apoptosis derives from experiments demonstrating that activation of endothelin type A receptor signaling attenuates apoptosis induced by β-adrenergic receptor agonists or cAMP. Furthermore, the anti-apoptotic effects of endothelin-1 are inhibited by PD098059, rapamycin, and wortmannin, consistent with the importance of PI3K and Akt in mediating the anti-apoptotic effects activated by the endothelin type A receptor (62).

The results described here using GLP-2 for analysis of GPCR signaling coupled to anti-apoptotic actions in BHK cells extend previous findings by demonstrating that GLP-2R signaling en-

hances cell survival via mechanisms that involve Bad and GSK-3 phosphorylation. Although we employed fibroblasts for these studies due to the lack of cell lines or enriched culture systems expressing the endogenous GLP-2R, several studies have demonstrated that activation of Bad and inhibition of GSK-3 are important for protection of endocrine cells and neurons *in vitro* (26, 29). We previously demonstrated that GLP-2 and forskolin attenuate cycloheximide-induced apoptosis and reduce caspase activation in a PKA-independent manner (13). Our current findings provide new evidence demonstrating that members of the glucagon-secretin receptor superfamily are capable of coupling to multiple signaling pathways for inhibition of cell death and that GLP-2R signaling converges on Bad and GSK-3 independently of Akt activation, resulting in enhanced cell survival. As both GLP-1 and GLP-2 modulate apoptotic pathways in a diverse number of cell types (8, 10, 11, 13, 63, 64), our current data illustrate the utility of examining GLP-2R signaling under a variety of conditions that induce cellular injury for delineation of the downstream anti-apoptotic effectors activated by GPCR signaling in diverse cell types.

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