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The Glucagon-like Peptide-2 Receptor Mediates Direct Inhibition of Cellular Apoptosis via a cAMP-dependent Protein Kinase-independent Pathway*

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Glucagon and the glucagon-like peptides regulate metabolic functions via signaling through a glucagon receptor subfamily of G protein-coupled receptors. Activation of glucagon-like peptide-2 receptor (GLP-2R) signaling maintains the integrity of the intestinal epithelial mucosa via regulation of crypt cell proliferation. Because GLP-2 decreases mortality and reduces intestinal apoptosis in rodents after experimental injury, we examined whether GLP-2R signaling directly modifies the cellular response to external injury. We show here that activation of GLP-2R signaling inhibits cycloheximide-induced apoptosis in baby hamster kidney fibroblasts expressing a transfected GLP-2 receptor. GLP-2 reduced DNA fragmentation and improved cell survival, in association with reduced activation of caspase-3 and decreased poly(ADP-ribose) polymerase cleavage and reduced caspase-8 and caspase-9-like activities. Both GLP-2 and forskolin reduced mitochondrial cytochrome *c* release and decreased the cycloheximide-induced cleavage of caspase-3 in the presence or absence of the PKA inhibitor H-89. Similarly, GLP-2 increased cell survival following cycloheximide in the presence of the kinase inhibitors PD98054 and LY294002. These findings provide evidence that signaling through G protein-coupled receptors of the glucagon superfamily is directly linked to regulation of apoptosis and suggest the existence of a cAMP-dependent protein kinase-, phosphatidylinositol 3-kinase-, and mitogen-activated protein kinase-independent pathway coupling GLP-2R signaling to caspase inhibition and cell survival.

Glucagon and the glucagon-like peptides are co-encoded within a common precursor, proglucagon, that is expressed in a tissue-specific manner, giving rise to glucagon in the pancreatic A cells and glucagon-like peptides-1 and 2 in the endocrine cells of the gastrointestinal tract (1, 2). Proglucagon-derived peptides play important roles in regulation of metabolic function following nutrient assimilation (2). Glucagon regulates hepatic glucose production and maintains plasma glucose in a narrowly defined physiological range by opposing the actions of insulin at the hepatocyte. Glucagon-like peptide-1 (GLP-1)[1] is secreted from the gut following nutrient ingestion and controls glycemia via stimulatory and inhibitory effects on insulin and glucagon, respectively (2, 3). In contrast, glucagon-like peptide-2 (GLP-2) exerts its principal actions on nutrient homeostasis proximal to nutrient absorption via regulation of the integrity of the mucosal epithelium (4, 5).

In addition to metabolic effects regulating fuel homeostasis, glucagon-like peptides also exert specific actions on cell proliferation and tissue regeneration. Glucagon potentiates proliferation of rat hepatocytes (6) and stimulates hepatic DNA synthesis following partial hepatectomy *in vivo* (7). GLP-1 increases islet proliferation in mice (8) and enhances islet regeneration and lowers blood glucose in rats following partial pancreatectomy (9). GLP-1 also appears to stimulate islet neogenesis via induction of pdx-1 expression in normal and diabetic rodents (10).

GLP-2 stimulates intestinal crypt cell proliferation in normal rats and mice leading to villus hyperplasia and expansion of the mucosal epithelium (4, 11, 12). The beneficial effects of GLP-2 in experimental models of intestinal injury have largely been attributed to enhancement of mucosal regeneration via GLP-2-dependent stimulation of crypt proliferation (13–16). Although GLP-2 inhibits apoptosis in the crypt compartment following administration of the nonsteroidal anti-inflammatory agent indomethacin (14), the mechanisms coupling GLP-2 signaling to anti-apoptotic effects in a direct or indirect manner remain unknown. Because intestinal cell lines expressing the endogenous GLP-2 receptor have not yet been identified, we have now examined the effects of GLP-2 receptor signaling on cell death in heterologous cells expressing the transfected rat GLP-2 receptor.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture medium, serum, and other supplements, including G418, were from Life Technologies, Inc. Cycloheximide, forskolin, protease inhibitor mixture (P-2714), and 4′,6-diamidino-2-phenylindole were purchased from Sigma. Recombinant human [Gly2]-GLP-2 was a kind gift from NPS Allelix Inc. (Mississauga, Canada). The caspase inhibitors Z-VAD-fmk and Z-YVAD-fmk and the kinase inhibitors H89 and LY294002 were obtained from Calbiochem (San Diego, CA). Ac-IETD-pNA and Ac-LEHD-pNA were from BIOSOURCE International (Camarillo, CA). PD98059 was obtained from New England

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[1] The abbreviations used are: GLP, glucagon-like peptide; GLP-2R, GLP-2 receptor; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; BHK, baby hamster kidney; PBS, phosphate-buffered saline; PARP, poly(ADP-ribose) polymerase; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid; PKA, cAMP-dependent protein kinase; PI, phosphatidylinositol; GPCR, G protein-coupled receptor; Erk, extracellular signal-regulated kinase; NGF, nerve growth factor; CHX, cycloheximide; pNA, *p*-nitroanilide.

Biolabs (Beverly, MA). X-gal was purchased from BioShop Canada Inc. (Burlington, Canada). All electrophoresis and immunoblotting reagents were purchased from Bio-Rad. The pCRE/β-galactosidase reporter plasmid (17) and the expression vector MtR(AB) (18) were gifts from R. D. Cone (Portland, OR) and G. S. McKnight (Seattle, WA), respectively.

Cell Culture, Apoptosis Induction, and Drug Treatments—Baby hamster kidney (BHK) fibroblasts containing the stably integrated pcDNA3.1 plasmid (BHK-pcDNA3) (Invitrogen, Carlsbad, CA) or the identical plasmid directing expression of the rat GLP-2 receptor (BHK-GLP-2R) were propagated as described previously (19). Stock cultures were trypsinized, and the cells were replated in culture medium lacking G418. Upon reaching 80–90% confluency, the cultures were starved in Dulbecco's modified Eagle's medium supplemented with 0.2% calf serum for 15–17 h prior to apoptosis induction by cycloheximide in the same medium, in the presence or absence of the indicated peptides or drugs for the indicated period of time. Adherent cells were then scraped off the culture dishes in PBS, combined with detached cells floating in the medium and spun at 12,000 rpm for 30 s. Depending on the type of assay to be performed, cell pellets were either stored at −70 °C or used immediately. Cycloheximide was dissolved in anhydrous ethyl alcohol, h[Gly2]-GLP-2 in PBS, pH 7.4, and forskolin, H89, LY294002, PD98059, Z-VAD-fmk, and Z-YVAD-fmk in dimethyl sulfoxide. Drug stock solutions were stored at −70 °C and diluted as required in their corresponding solvents, immediately before being added either alone or in combination to the cultures at the desired final concentrations. Control cultures were subjected to the same manipulations as treated cells but in the absence of the drugs. Ethanol and dimethyl sulfoxide final concentrations were identical in every culture irrespective of the particular treatment group.

Microscopy—Morphological assessment of cell death was performed by examination of the cultures under phase contrast microscopy for the presence of cell detachment and the appearance of plasma membrane blebbing. Phase contrast as well as bright field microscopy were performed on a PhotoZoom Leica inverted microscope. Images of representative areas of the cultures were acquired with a JVC TK-1280U color video camera and the Leica Q500MC Image Analysis System. To visualize apoptotic nuclear morphology and chromatin condensation, cultures were fixed with 2% paraformaldehyde and then stained with 1 μg/ml 4′,6-diamidino-2-phenylindole in PBS. The cells were washed once with PBS before mounting with 50% glycerol and viewed under a Olympus BX60 System fluorescence microscope. Images were recorded using a CoolSNAP CCD video camera (Photometrics GmbH, Munich) and CoolSNAP software.

Analysis of Genomic DNA Fragmentation by Agarose Gel Electrophoresis—For the detection of DNA fragmentation associated with apoptosis, genomic DNA was extracted from lysed cells and treated with proteinase K and RNase A, according to standard protocols. DNA samples were size fractionated by electrophoresis in 1.5% agarose gel in 1× TAE buffer, and visualized by UV light after ethidium bromide staining. Densitometry was performed on Polaroid pictures of the ethidium bromide stained gels using a Hewlett Packard ScanJet 3p scanner and the NIH Image software.

Cell Viability Assay—Cells were exposed to either vehicle alone or cycloheximide in the presence or absence of the indicated drugs and inhibitors. At the indicated periods of time following apoptosis induction, the number of viable cells in each condition was assessed by measuring the bioreduction of a MTS tetrazolium salt at 490 nm using the CellTiter 96 aqueous assay (Promega, Madison, WI).

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis—Cell pellets obtained as indicated above were lysed at 4 °C in PBS containing 1% Triton X-100 and protease inhibitor mixture (1:100 dilution). Lysates were cleared at 12,000 rpm for 15 min at 4 °C, treated for 60 min at 35 °C with sample buffer containing β-mercaptoethanol and stored at −70 °C until use. Protein concentration was determined using a Coomassie dye assay (Bio-Rad) and bovine serum albumin as a standard. 40 μg of cell lysate were separated by discontinuous SDS-polyacrylamide gel electrophoresis under reducing conditions and electrotransferred onto Hybond-C nitrocellulose membrane (Amersham Pharmacia Biotech) using standard procedures. The resulting blot was blocked with 5% skim milk in PBS containing 0.2% Tween 20 and incubated with a designed primary antibody overnight at room temperature. Proteins were detected with a secondary antibody conjugated to horseradish peroxidase and an enhanced chemiluminescence commercial kit (Amersham Pharmacia Biotech). Primary antibodies reactive to procaspase-3/active caspase-3 p17 subunit (1:5,000 dilution, gift of RP Sekaly, Montreal, PQ), poly(ADP-ribose) polymerase (PARP) (1:4,000 dilution; Pharmingen Canada Inc., Mississauga, Canada), cytochrome *c* (1 μg/ml; BIOSOURCE International), porin/VDAC 31HL (2 μg/ml; Calbiochem), phospho-Erk1/Erk2, Erk1/Erk-2, phospho-Akt, Akt (1:1,000; New England Biolabs), and anti-actin (1:5,000 dilution; Sigma) were used in this study. The anti-actin polyclonal antibody was utilized to monitor loading and transfer conditions.

Caspase Activity Assay—Caspase enzymatic activity was assessed by the cleavage of site-selected tetrapeptide chromogenic reporter substrates with the specificity of IETD (caspase-8) and LEHD (caspase-9-like). Cells were lysed at 4 °C in 50 mM Hepes-KOH, pH 7.4, 1 mM EDTA buffer containing 75 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, and 100 KIU/ml aprotinin (Trasylol, Bayer) and spun at 12,000 rpm for 20 min at 4 °C, and the supernatant was recovered. Enzymatic reactions (final volume, 0.25 ml) were performed at 37 °C and contained 50–75 μg of cell lysate and 100 μM of chromogenic reporter substrate in 50 mM Hepes-KOH, pH 7.4, buffer containing 75 mM NaCl, 2 mM dithiothreitol, and 0.1% CHAPS. Caspase catalyzed release of the chromophore *p*-nitroanilide was monitored spectrophotometrically at 405 nm. Optical density readings were corrected for background and standardized to equivalent protein.

Apoptosis Assessment in Transfected Cells—Transient transfection of BHK-GLP-2R cells was done by calcium phosphate coprecipitation as described previously (19). Cell cultures were transfected with 6.5 μg of a RSV-β-galactosidase expression plasmid, as a marker for transfection, plus 13.5 μg of pBluescript II (Stratagene, La Jolla, CA) carrier DNA or with the β-galactosidase marker plasmid plus 10 μg of MtR(AB) expression vector and carrier DNA for a total of 20 μg. After glycerol shock, cells were cultured in serum-depleted medium for 15–17 h (Dulbecco's modified Eagle's medium with 0.2% calf serum), induced to undergo apoptosis by cycloheximide in the presence or absence of the indicated drugs, and then fixed in 2% paraformaldehyde. Transfected, β-galactosidase-expressing cells were identified by histochemistry with X-gal (2 mg/ml in PBS containing 5 mM potassium ferrycianide, 5 mM potassium ferrocyanide, and 100 mM $MgCl_2$). The percentage of β-galactosidase-positive cells remaining in the plate that showed evidence of apoptosis was determined by counting >200 cells from at least 15–20 different fields. Transfected cells were scored as apoptotic when appearing rounded-up with a shrunken morphology, as opposed to the flat extended appearance of viable, healthy cells (see Fig. 5*C*, *upper right panel*).

cAMP-dependent Protein Kinase Activity Measurement—The extent of PKA inhibition achieved by the transfected MtR(AB) dominant negative expression plasmid was assessed indirectly by determining its ability to inhibit both h[Gly2]-GLP-2 and forskolin-induced transcriptional activation of a CRE/β-galactosidase reporter plasmid, as described previously (19). The inhibitory effect of H89 on both basal and h[Gly2]-GLP-2 or forskolin-induced cAMP-dependent protein kinase activity was measured *in vitro* using a fluorescent-labeled kemptide (PepTag nonradioactive cAMP-dependent protein kinase assay; Promega). Phosphorylation of the kemptide alters the net charge of the peptide, allowing the phosphorylated form to be separated from the nonphosphorylated form on an agarose gel at pH 8. PKA activity was defined as kemptide phosphorylation that could be inhibited by 10 μM protein kinase inhibitor-(5–22)-amide peptide (Upstate Biotechnology, Lake Placid, NY).

Mitochondria and Cytosol Isolation—Cells were resuspended in ice-cold mitochondrial isolation buffer (10 mM Hepes-KOH, pH 7.4, 200 mM mannitol, 70 mM sucrose, 1 mM EGTA, and protease inhibitor mixture (1:100 dilution)) and lysed at 4 °C with a glass Dounce homogenizer. After pelleting nuclei and unbroken cells at 770 × *g* for 10 min, the supernatant was centrifuged for 15 min at 10,000 × *g* to collect the heavy membrane fraction enriched for mitochondria. The newly obtained supernatant containing the cytosol was further centrifuged for 15 min at 20,800 × *g* to remove the light membrane fraction and then stored at −70 °C. The mitochondria-enriched pellet was washed once in mitochondrial isolation buffer and then resuspended in the same buffer and stored at −70 °C until use. Protein concentration in both cytosolic and mitochondrial fractions was determined before preparing the samples for immunoblot analysis as described above.

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

RESULTS

The glucagon-like peptide-2 receptor is expressed in a highly tissue-specific pattern primarily in the intestine and brain (20). Despite ongoing attempts by our laboratory and others, intestinal cell lines that express the endogenous GLP-2 receptor

have not yet been identified. Accordingly we have studied GLP-2R signaling and its putative coupling to anti-apoptotic pathways in BHK fibroblasts stably transfected with the rat GLP-2 receptor (19). Incubation of wild type BHK cells or BHK-GLP-2R cells with cycloheximide alone at concentrations (5–80 μM) that inhibit protein synthesis by greater than 95% produced a visibly detectable decrease in cell viability. In contrast, incubation of cells with cycloheximide and h[Gly2]-GLP-2 or forskolin (or 8-bromo-cyclic AMP) resulted in significantly improved cell survival (Fig. 1, *A* and *B*). Furthermore, 1,9-dideoxyforskolin, a forskolin analog that does not stimulate adenylate cyclase, had no effect on the cycloheximide-induced decrease in cell viability (data not shown). Cycloheximide-induced cell death was reduced by the pancaspase inhibitor Z-VAD-fmk but not by Z-YVAD-fmk, an inhibitor of caspase-1-like activity (Fig. 1*C*). The extent of cycloheximide-induced cell death was markedly attenuated in the presence of $ZnCl_2$, a known inhibitor of caspase-3-mediated apoptosis (21). Morphologically, BHK-GLP-2R cells exhibited characteristic nuclear features consistent with apoptosis including chromatin condensation and margination as well as nuclear fragmentation following treatment with cycloheximide (Fig. 1*D*). DNA fragmentation was induced by cycloheximide and reduced by treatment of cells with h[Gly2]-GLP-2 (Fig. 1*E*). Taken together, these findings are consistent with the cycloheximide-dependent activation of apoptosis in BHK-GLP-2R cells *in vitro*.

Consistent with the importance of caspase activation for cycloheximide-induced cellular injury, the levels of the p17 active subunit of caspase-3 were increased in a time-dependent manner following treatment with cycloheximide and reduced following treatment of cells with either h[Gly2]-GLP-2 or forskolin (Fig. 2*A*). Similarly, the cycloheximide-induced cleavage of PARP was clearly attenuated following treatment with either h[Gly2]-GLP-2 or forskolin (Fig. 2*A*). In contrast, the protective effects of h[Gly2]-GLP-2 but not forskolin on both caspase-3 and PARP cleavage were absent in control BHK-pcDNA3 cells (Fig. 2*B*), consistent with the importance of the GLP-2R for transduction of the GLP-2-mediated signal to downstream apoptosis pathways.

Because caspase-3 processing and PARP cleavage are distal events that follow activation of upstream signaling molecules, we assessed the effects of GLP-2 on the activation of more proximal caspase enzymes following cycloheximide treatment of BHK cells. Analysis of initiator caspase activity using synthetic tetrapeptide substrates that are preferentially cleaved by caspase-8 (IETD-pNA) or caspase-9-like (LEHD-pNA) proteases showed that both h[Gly2]-GLP-2 and forskolin significantly inhibited the induction of IETD-pNA and LEHD-pNA cleaving activity following cycloheximide treatment of BHK-GLP-2R cells (Fig. 3). In contrast, only forskolin, but not h[Gly2]-GLP-2 inhibited activation of caspase-8- and caspase-9-like activity in control BHK-pcDNA3 cells (Fig. 3), consistent with the importance of the GLP-2R for transduction of the anti-apoptotic effects of h[Gly2]-GLP-2 *in vitro*. The specificity of caspase induction was further illustrated by findings that caspase-1-like activity, as assessed using YVAD-pNA as a substrate, was not induced following treatment of BHK cells with similar concentrations of cycloheximide (data not shown).

The finding that GLP-2R signaling was coupled to inhibition of cycloheximide-induced caspase-9-like activity prompted us to assess mitochondrial cytochrome *c* release, a known upstream effector of caspase-9 activation (22). Cycloheximide treatment of BHK-GLP-2R cells was associated with a marked time-dependent induction in the levels of cytosolic cytochrome *c* (Fig. 4). Both h[Gly2]-GLP-2 and forskolin markedly attenuated the cycloheximide induction of cytosolic cytochrome *c* release from BHK-GLP-2R cells (Fig. 4). Because both GLP-2 and forskolin are known to exert their effects via activation of adenylate cyclase and downstream PKA-dependent signaling pathways (19, 20), we assessed the importance of PKA signaling for cellular apoptosis in the presence or absence of H-89, a pharmacological inhibitor of protein kinase A. H-89 alone reduced cell viability in BHK-GLP-2R cells, consistent with the importance of basal PKA signaling for cell survival *in vitro* (Fig. 5*A*). However, both h[Gly2]-GLP-2 and forskolin significantly increased cell survival after cycloheximide in the presence of H-89 (Fig. 5*A*), demonstrating that stimulation of PKA-dependent signaling pathways is not required for the anti-apoptotic actions of these agents (Fig. 5*A*). To verify that H-89 inhibited PKA activity in BHK-GLP-2R cells, we assessed basal and stimulated PKA activity following incubation of cells with h[Gly2]-GLP-2 or forskolin. PKA activity was induced following treatment of BHK-GLP-2R cells with h[Gly2]-GLP-2 or forskolin, and PKA activation by these agents was effectively blocked by H-89 in the same experiments (Fig. 5*A*).

These observations clearly suggest that h[Gly2]-GLP-2 inhibits cycloheximide-induced apoptosis in BHK-GLP-2R cells in a PKA-independent manner. Consistent with these findings, both h[Gly2]-GLP-2 and forskolin attenuated the cycloheximide-induced cleavage of procaspase-3 to the active caspase-3 p17 subunit in H-89-treated BHK-GLP-2R cells (Fig. 5*B*). To provide complementary evidence for the PKA-independent actions of GLP-2 on apoptotic pathways, PKA activity was inhibited by transfection of MtR(AB), a plasmid encoding the dominant negative regulatory subunit of protein kinase A (18). Analysis of transfected cells following cycloheximide treatment demonstrated that both h[Gly2]-GLP-2 and forskolin produced a significant reduction in the number of apoptotic cells despite transfection of MtR(AB) (Fig. 5*C*). In contrast, the activity of a PKA-dependent reporter gene, CRE-β-galactosidase, was markedly stimulated by h[Gly2]-GLP-2 or forskolin, and the stimulatory activity was significantly reduced in MtR(AB)-transfected cells (Fig. 5*C*).

Because activation of both the glucagon and GLP-1 receptors has been associated with induction of mitogen-activated protein kinase activity (23, 24), we assessed the effects of PD98059, a known inhibitor of Erk1/2 activity, on cycloheximide-induced apoptosis. Incubation of BHK-GLP-2R cells with PD98059 alone had no effect on the number of viable cells in the presence or absence of cycloheximide, demonstrating that basal Erk 1/2 activity is not required for cell survival in BHK-GLP-2R cells (Fig. 6*A*). Furthermore, both h[Gly2]-GLP-2 and forskolin significantly increased cell viability following cycloheximide in the presence of PD98059. In contrast, the activation of phosphorylated Erk1/2 by lysophosphatidic acid or fetal calf serum was markedly attenuated in the presence of PD98059 (Fig. 6*A*). These findings demonstrate that the effects of GLP-2R signaling on cell survival following cycloheximide treatment are independent of Erk1/2 activation in BHK-GLP-2R cells.

Because GLP-1 activates phosphatidylinositol 3-kinase (PI 3-kinase) in INS-1 cells (25) and PI 3-kinase is known to exert anti-apoptotic effects via induction of Akt (26), we examined whether the anti-apoptotic effects of GLP-2 might be mediated via a PI 3-kinase-dependent pathway. Treatment of BHK cells with LY294002, an inhibitor of phosphatidylinositol 3-kinase signaling, significantly reduced BHK cell survival, and co-incubation of cells with both LY294002 and cycloheximide produced a further diminution in survival beyond that seen with either agent alone (Fig. 6*B*). In contrast, cell survival was significantly increased in cycloheximide-treated cells incubated with either h[Gly2]-GLP-2 or forskolin in the presence of

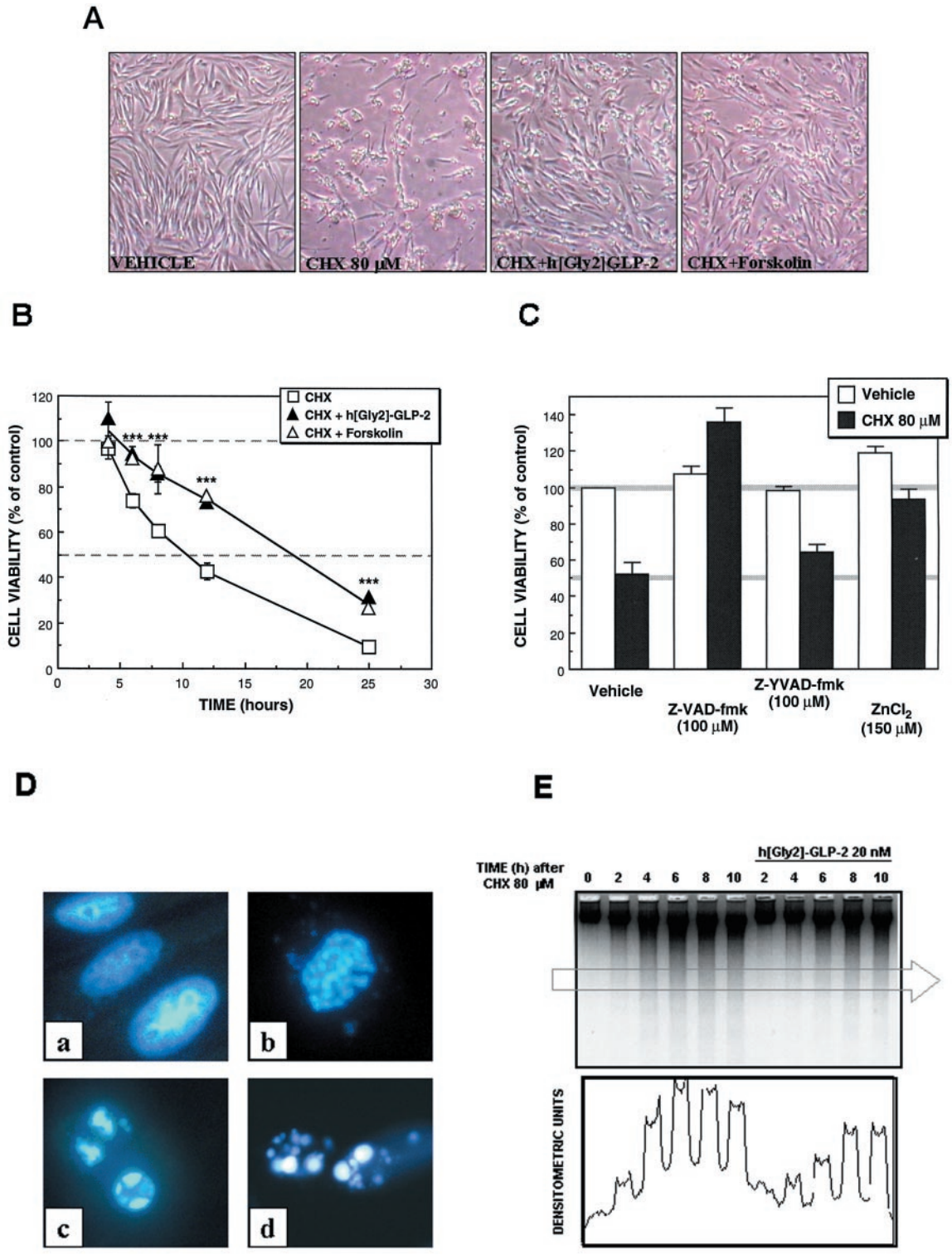

FIG. 1. **GLP-2 and forskolin protect BHK-GLP-2R cells from cycloheximide-induced apoptosis.** *A*, phase contrast micrographs showing the appearance of untreated control cultures and of cultures exposed for 8 h to 80 μM CHX alone or in combination with 20 nM h[Gly2]-GLP-2 or 20 μM forskolin. Magnification, $\times$100. *B*, for assessment of cell viability, cells were cultured as described under "Experimental Procedures" and treated with 80 μM CHX in the presence or absence of 20 nM h[Gly2]-GLP-2 or 20 μM forskolin. Cell viability at different time points was quantified using a tetrazolium salt bioreduction assay and expressed as percentage of the values obtained from analysis of vehicle alone-treated control cultures. Data shown are the means $\pm$ S.D. from four to six independent experiments, each one performed in quadruplicate. ***, $p < 0.001$, CHX plus either h[Gly2]-GLP-2 or forskolin *versus* CHX alone. *C*, effect of caspase inhibitors on cell viability. BHK-GLP-2R cells were pretreated with the indicated caspase inhibitors for 45 min prior to CHX 80 μM or vehicle alone for 8 h. Cell viability was then determined as described for *B*. Data are the means $\pm$ S.D. ($n = 4$). *D*, changes in nuclear morphology and chromatin condensation following apoptosis induction by CHX. Cells were exposed to CHX 80 μM (*panels b–d*) or vehicle alone (*panel a*) for 8 h. Cell nuclei were visualized using fluorescence microscopy after 4′,6-diamidino-2-phenylindole staining. Magnification $\times$ 1000. *E*, agarose gel electrophoresis of DNA extracted from cells exposed to CHX 80 μM for 0–10 h in the absence or presence of h[Gly2]-GLP-2. The densitometric profile of the gel along the *horizontal axis*, as indicated by the *arrow*, is shown in the *lower panel* to provide semiquantitative information on the extent of chromatin fragmentation illustrated in the *upper panel*. Results are representative of two independent experiments.

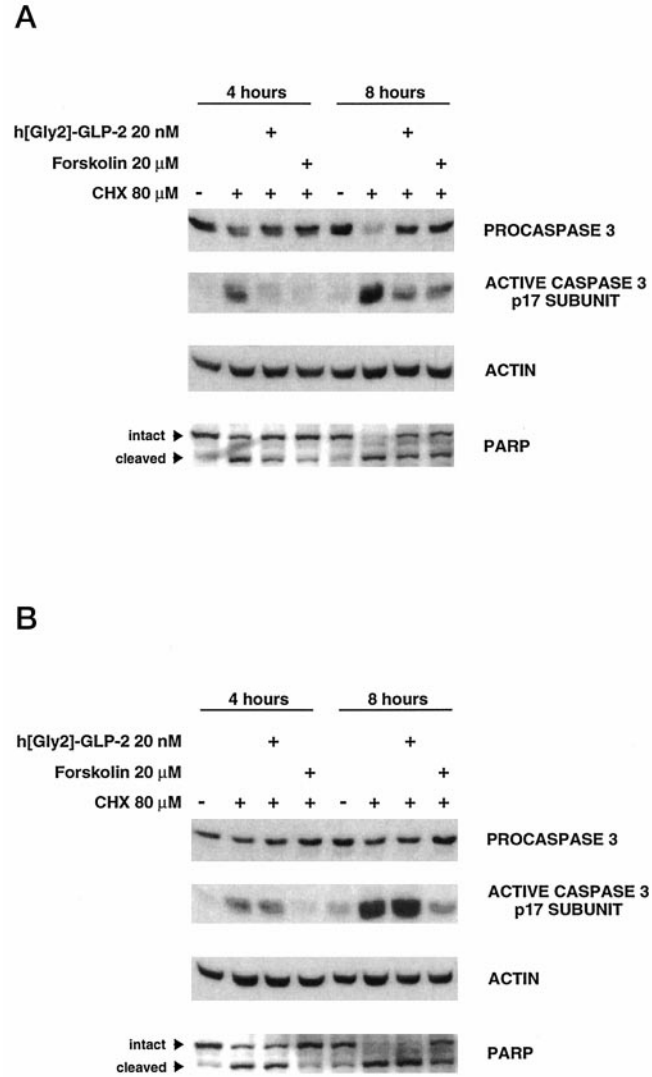

FIG. 2. **Effect of h[Gly2]-GLP-2 or forskolin on CHX-induced caspase-3 processing and PARP cleavage in BHK-GLP-2R and BHK-pcDNA3 cells.** BHK-GLP-2R (*A*) and BHK-pcDNA3 cells (*B*) were serum-starved for 15 h and then treated with CHX in the presence or absence of h[Gly2]-GLP-2 or forskolin for 4 or 8 h as indicated. Cell extracts were then analyzed by immunoblotting for caspase-3 and PARP cleavage as described under "Experimental Procedures." Equal loading was verified by reprobing the blots with an anti-actin antibody. Results are representative of four independent experiments.

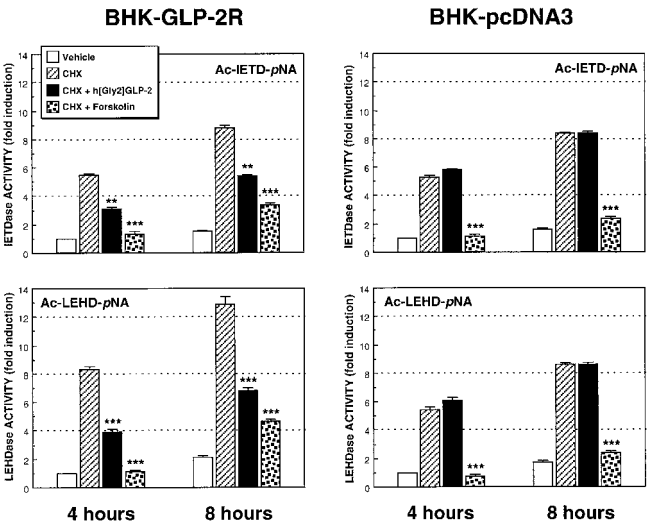

FIG. 3. **Effect of h[Gly2]-GLP-2 or forskolin on CHX-induced caspase-8 and caspase-9 enzymatic activity in BHK-GLP-2R and BHK-pcDNA3 cells.** BHK-GLP-2R and BHK-pcDNA3 cells were treated with CHX 80 μM in the presence or absence of 20 nM h[Gly2]-GLP-2 or 20 μM forskolin. At the indicated times, cell lysates were prepared and used to determine caspase enzymatic activity by measuring the release of the pNA chromophore from peptide substrates selective for caspase-8 (IETD) or caspase-9-like (LEHD) proteases. Enzymatic activity is expressed as fold induction relative to the activity in vehicle-treated cultures at 4 h. Data are the means ± S.D. of triplicate determinations from one representative experiment of three with similar results. ** and ***, $p < 0.01$ and $p < 0.001$, respectively, CHX plus either h[Gly2]-GLP-2 or forskolin *versus* CHX alone.

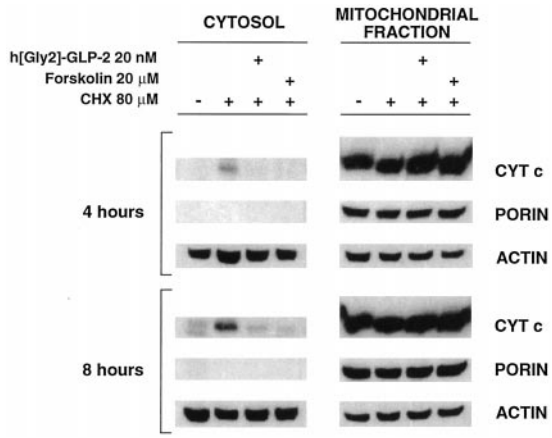

FIG. 4. **h[Gly2]-GLP-2 or forskolin prevents cycloheximide-induced release of mitochondrial cytochrome *c* into the cytosol in BHK-GLP-2R cells.** BHK-GLP-2R cells were exposed to CHX in the presence or absence of h[Gly2]-GLP-2 or forskolin. After 4 and 8 h, mitochondrial pellets and cytosolic supernatants were prepared and Western blot analysis was performed to detect cytochrome *c* (*CYT c*) in both subcellular fractions. The quality of the subcellular fractionation and the equivalent protein loading per lane were monitored by probing the blots for porin and actin, respectively.

LY294002 at concentrations that completely block Akt activation by either lysophosphatidic acid or fetal calf serum (Fig. 6*B*). These results imply that although basal PI 3-kinase activity is required for BHK-GLP-2R cell survival, the effects of h[Gly2]-GLP-2 on cycloheximide-induced cell death are mediated through a phosphatidylinositol 3-kinase-independent pathway.

DISCUSSION

Although signaling through G protein-coupled receptors (GPCRs) of the glucagon/GLP-1/GLP-2 receptor superfamily has not previously been reported to directly modify apoptotic pathways, recent experiments provide increasing evidence linking GPCR activation and signal transduction pathways modulating cell death. Activation of the somatostatin receptor modulates pH-dependent apoptosis in heterologous cell types (27, 28) and signaling through the PTH/PTHrP (parathyroid hormone/parathyroid hormone-related protein) receptor activates apoptotic pathways in cells of the chondrocyte and osteoblast lineages (29). Activation of the pituitary adenylate cyclase-activating peptide receptor prevented apoptosis in cerebellar neurons, and the effects of pituitary adenylate cyclase-activating peptide signaling on apoptosis were reversed following transfection of cells with a dominant negative inhibitor of protein kinase A (30), consistent with the importance of the PKA pathway for antiapoptotic action in this cell type. In contrast, our data clearly show that the antiapoptotic effects of GLP-2 are PKA-independent. Similarly, stimulation of thymocyte apoptosis following activation of β-adrenergic receptor signaling is mediated by a PKA-independent, $G_s\alpha$-dependent pathway (31). These findings illustrate the diversity of signal-

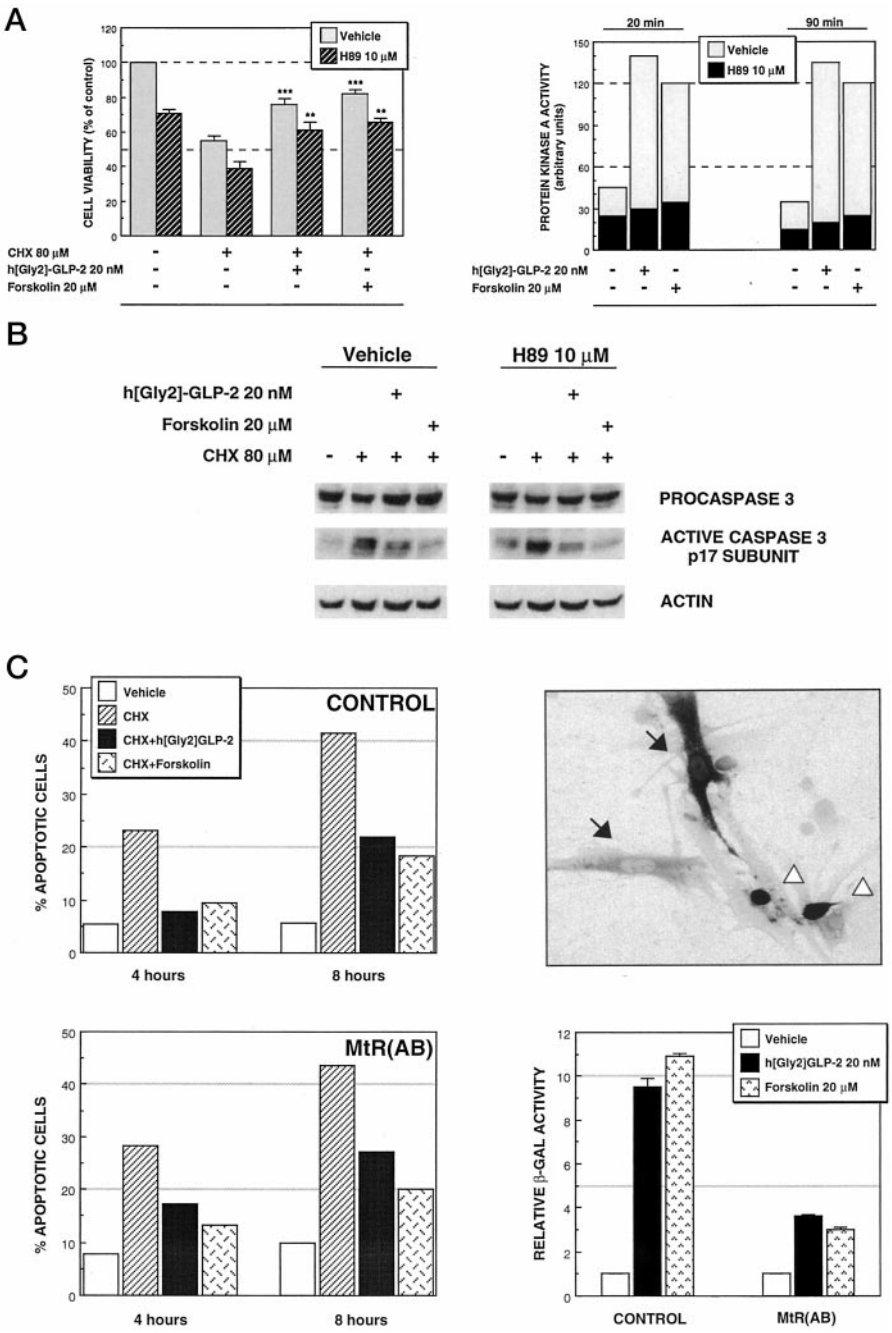

FIG. 5. **GLP-2 and forskolin protect BHK-GLP-2R cells from cycloheximide-induced apoptosis in a PKA-independent manner.** *A*, *left panel*, cultures were pretreated with H89 or vehicle alone for 1 h prior to CHX in the presence or absence of h[Gly2]-GLP-2 or forskolin. After 8 h, cell viability was determined as described for Fig. 1*B* and expressed as a percentage of the values for vehicle alone-treated control cells not exposed to CHX. Data are the means ± S.D. from three independent experiments, each one performed in quadruplicate. ** and ***, $p < 0.01$ and $p < 0.001$, respectively, CHX plus either h[Gly2]-GLP-2 or forskolin *versus* the corresponding CHX alone-treated cultures. *Right panel*, BHK-GLP-2R cells were pretreated with H89 or vehicle alone for 1 h and then exposed to vehicle alone or either h[Gly2]-GLP-2 or forskolin for 20 and 90 min. PKA activity in cell extracts was determined using a fluorescent kemptide assay as detailed under "Experimental Procedures." Results are representative of two independent experiments performed in duplicate. *B*, Western blot analysis of cell extracts from control or H-89-treated BHK-GLP-2R cells treated as described for Fig. 5*A*. Extracts were analyzed by immunoblotting for caspase-3 activation or actin. *C*, cells were transiently transfected with RSV-β-galactosidase either alone (control, *upper left panel*) or in combination with a dominant negative PKA mutant expression plasmid (MtR(AB), *lower left panel*). After 16 h, cells were treated with CHX 80 μM in the presence or absence of 20 nM h[Gly2]-GLP-2 or 20 μM forskolin. After 4 and 8 h of CHX treatment, cultures were fixed, stained for β-galactosidase expression, and transfected flat (healthy, *arrows* in *upper right panel*) and round (apoptotic, *open arrowheads* in *upper right panel*) blue cells were counted. The data shown represent the percentage of apoptotic cells. A representative micrograph (400× magnification) of cells transfected with the β-galactosidase reporter plasmid alone and treated with CHX 80 μM for 8 h is shown in the *upper right panel* after histochemistry with X-gal. *Arrows* point to healthy β-galactosidase positive cells. *Open arrowheads* point to apoptotic β-galactosidase positive cells. *Lower right panel*, h[Gly2]-GLP-2 or forskolin-induced transcriptional activation of a pCRE/β-galactosidase reporter plasmid in the absence (control) or presence of the cotransfected MtR(AB) expression plasmid. Cells were treated for 8 h with h[Gly2]-GLP-2 or forskolin and then assayed for β-galactosidase activity. Reporter gene activity is expressed as fold induction *versus* vehicle-treated cells following normalization for protein content. Data are the means ± S.D. from two independent experiments each one performed in triplicate.

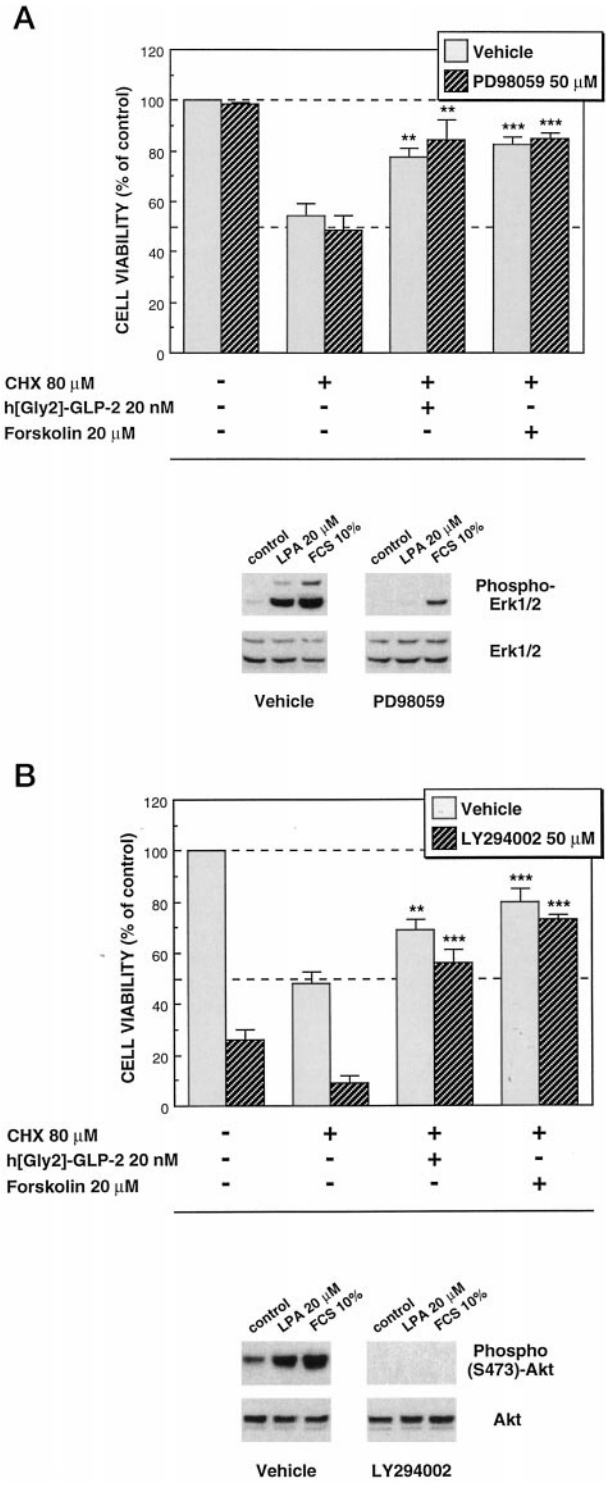

FIG. 6. **Protection from cycloheximide-induced cell death by GLP-2 or forskolin in BHK-GLP-2R cells is not suppressed by pharmacological inhibition of either MEK or PI 3-kinase.** BHK-GLP-2R cells were pretreated with either vehicle alone or the MEK inhibitor PD98059 (*A*) or the PI 3-kinase inhibitor LY294002 (*B*) for 1 h prior to CHX treatment in the presence or absence of h[Gly2]-GLP-2 or forskolin. After 8 h, cell viability was determined and expressed as a percentage of the values from control cultures not exposed to CHX. Data are the means ± S.D. from two independent experiments each one performed in quadruplicate. ** and ***, $p < 0.01$ and $p < 0.001$, respectively, CHX plus either h[Gly2]-GLP-2 or forskolin *versus* the corresponding CHX alone-treated cultures. Representative Western blots of phosphorylated Erk1/Erk2 (*A*, *bottom*) and phosphorylated Akt (*B*, *bottom*) in cells incubated with vehicle (control) or either 10% fetal calf serum or 20 μM lysophosphatidic acid (*LPA*) for 5 min following a 60-min pretreatment with 50 μM PD98059 (*A*) or 50 μM LY294002 (*B*) are shown. Anti-Erk1/2 and anti-Akt polyclonal antibodies were used to monitor loading and transfer conditions.

ing pathways downstream of GPCR activation and emphasize that GPCRdependent effects on apoptotic pathways are highly cell- and receptor-specific.

GPCR-dependent modulation of cell death has been extensively studied in the heart, and stimulation of cardiac myocytes with β-adrenergic agonists such as isoproterenol leads to induction of apoptosis that is prevented by inhibitors of protein kinase A (32). Similarly, stimulation of α-adrenergic receptor signaling with agents such as norepinephrine also leads to cardiomyocyte apoptosis, and these effects were abolished by co-incubation with the protein kinase A inhibitor H-89 (33). Although adrenergic stimulation leads to activation of Erk1/2 in rat ventricular myocytes, inhibition of the Erk1/2 pathway had no effect on adrenergic receptor-mediated apoptosis (34), in agreement with our findings that Erk1/2 activation was not essential for GLP-2 inhibition of apoptosis in BHK-GLP-2R cells.

Although receptors for glucagon, GLP-1, and GLP-2 classically transduce their effects via activation of adenylate cyclase and protein kinase A (20, 35, 36), studies of both glucagon and GLP-1 action have provided evidence for PKA-independent signaling following activation of their cognate receptors (4, 12, 23, 25, 37). Similarly, our data clearly demonstrate that although GLP-2 activates a PKA-dependent pathway in transfected fibroblasts expressing the human or rat GLP-2 receptor (19, 20), the effects of GLP-2R signaling on cell survival and downstream caspase activation are mediated via PKA-independent pathways. The observations that GPCR ligands may also activate metalloproteinase activity leading to transactivation of growth factor receptor activity (38) further expands the complexity of intracellular signaling pathways residing downstream of GPCR activation.

In previous studies of GLP-2 action in BHK-GLP-2R cells in the absence of cycloheximide-induced cellular injury, we did not detect GLP-2-dependent activation of intracellular calcium influx, and GLP-2 stimulation failed to activate Erk1/2, p70 S6 kinase or Akt1 kinase activity (19). The findings on the lack of Erk induction in BHK-GLP-2R cells differ from studies of NGF action in PC 12 cells, where activation of Erk activity prevents apoptosis induced by NGF withdrawal (39). Similarly, the PI 3-kinase pathway is required for the antiapoptotic effects of NGF in PC 12 cells because PI 3-kinase inhibitors such as LY294002 also inhibit the effects of NGF on cell survival (26). In contrast, activation of GLP-2R signaling reduced cell death and decreased caspase activation in the presence of H-89 or MtR(AB), LY294002, or PD98059. Hence, although elevated levels of cAMP and/or PKA may negatively regulate apoptosis in some cell types (40), the available evidence from our studies points to the existence of a GLP-2-dependent, PKA-independent pathway coupled to inhibition of caspase-3 cleavage and prevention of cell death. Similarly, the lack of effect of the inhibitors LY294002 and PD98059 on GLP-2-mediated enhancement of cell survival strongly suggest that the anti-apoptotic effects of GLP-2R are mediated via a PI 3-kinase- and mitogen-activated protein kinase-independent pathway.

The cellular targets utilized by cycloheximide for induction of cell death appear cell type-specific, remain incompletely understood, and may involve both mitochondrial and death receptor-associated pathways. Although activation of Fas leading to caspase-8 cleavage and caspase-3 activation may occur independent of the mitochondrial pathway, recent evidence suggests that caspase-8 activation may also lead to mitochondrial cytochrome *c* release via a cycloheximide-sensitive pathway in

specific cell types (41). Although Fas ligand was not expressed in cycloheximide-sensitive Jurkat cells prior to or following induction of apoptosis, a dominant negative Fas-associated death domain protein completely inhibited cycloheximide-induced apoptosis, strongly implicating the importance of the Fas-associated death domain adapter protein in cycloheximide-induced cell death (42). The observation that GLP-2 inhibits the cycloheximide induction of both cytochrome *c* release and IETD-pNA and LEHD-pNA cleaving activity is consistent with previous studies of cycloheximide action (41) and suggests that GLP-2R signaling may interact with multiple signaling pathways upstream of caspase-3 cleavage.

GLP-2 ameliorates experimental intestinal injury in rats following nutritional deprivation, intestinal resection, and vascular ischemia (15, 16, 43) and in mice following chemically induced injury to the large and small bowel (13, 14). Although the beneficial effects of GLP-2 on intestinal mucosa were originally attributed to stimulation of crypt cell proliferation (4, 12, 23), we detected reduced apoptosis in the crypt compartment following GLP-2 treatment of mice with intestinal injury (14). Because the cellular localization of intestinal GLP-2 receptor expression has not yet been identified, our data generated in a fibroblast cell line may not necessarily be directly applicable to GLP-2 action in the intestine *in vivo*. Nevertheless, the observations that the transfected GLP-2 receptor confers resistance to caspase activation and apoptosis in heterologous cells in the presence of GLP-2 clearly suggest that intestinal cells expressing the endogenous GLP-2R may also be protected from cell death associated with exposure to genotoxic stress *in vivo*. The finding that GLP-2 improves cell survival and reduces caspase activation provides new evidence linking direct activation of GLP-2 receptor signaling to signaling pathways regulating apoptosis *in vitro*. Identification of the signaling mechanisms underlying the coupling of the GLP-2 receptor to inhibition of caspase activity and enhanced cell survival may facilitate identification of targets for prevention of cell death in the mucosal epithelium of the gastrointestinal tract.

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REFERENCES

1. Mojsov, S., Heinrich, G., Wilson, I. B., Ravazzola, M., Orci, L., and Habener, J. F. (1986) *J. Biol. Chem.* **261,** 11880–11889
2. Drucker, D. J. (1998) *Diabetes* **47,** 159–169
3. Kieffer, T. J., and Habener, J. F. (1999) *Endocr. Rev.* **20,** 876–913
4. Drucker, D. J., Ehrlich, P., Asa, S. L., and Brubaker, P. L. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 7911–7916
5. Drucker, D. J. (1999) *Trends Endocrinol. Metab.* **10,** 153–156
6. Kimura, M., and Ogihara, M. (1997) *Eur. J. Pharmaco.* **324,** 267–276
7. Bucher, N. L., and Swaffield, M. N. (1975) *Adv. Enzyme Regul.* **13,** 281–293
8. Edvell, A., and Lindstrom, P. (1999) *Endocrinology* **140,** 778–783
9. Xu, G., Stoffers, D. A., Habener, J. F., and Bonner-Weir, S. (1999) *Diabetes* **48,** 2270–2276
10. Stoffers, D. A., Kieffer, T. J., Hussain, M. A., Drucker, D. J., Egan, J. M., Bonner-Weir, S., and Habener, J. F. (2000) *Diabetes* **49,** 741–748
11. Drucker, D. J., Shi, Q., Crivici, A., Sumner-Smith, M., Tavares, W., Hill, M., Deforest, L., Cooper, S., and Brubaker, P. L. (1997) *Nature Bio/Technology* **15,** 673–677
12. Tsai, C.-H., Hill, M., Asa, S. L., Brubaker, P. L., and Drucker, D. J. (1997) *Am. J. Physiol.* **273,** E77–E84
13. Drucker, D. J., Yusta, B., Boushey, R. P., Deforest, L., and Brubaker, P. L. (1999) *Am. J. Physiol.* **276,** G79–G91
14. Boushey, R. P., Yusta, B., and Drucker, D. J. (1999) *Am. J. Physiol.* **277,** E937–E947
15. Scott, R. B., Kirk, D., MacNaughton, W. K., and Meddings, J. B. (1998) *Am. J. Physiol.* **275,** G911–G921
16. Prasad, R., Alavi, K., and Schwartz, M. Z. (2000) *J. Pediatr. Surg.* **35,** 357–359
17. Chen, W., Shields, T. S., Stork, P. J., and Cone, R. D. (1995) *Anal. Biochem.* **226,** 349–354
18. Correll, L. A., Woodford, T. A., Corbin, J. D., Mellon, P. L., and McKnight, G. S. (1989) *J. Biol. Chem.* **264,** 16672–16678
19. Yusta, B., Somwar, R., Wang, F., Munroe, D., Grinstein, S., Klip, A., and Drucker, D. J. (1999) *J. Biol. Chem.* **274,** 30459–30467
20. Munroe, D. G., Gupta, A. K., Kooshesh, P., Rizkalla, G., Wang, H., Demchyshyn, L., Yang, Z.-J., Kamboj, R. K., Chen, H., McCallum, K., Sumner-Smith, M., Drucker, D. J., and Crivici, A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 1569–1573
21. Perry, D. K., Smyth, M. J., Stennicke, H. R., Salvesen, G. S., Duriez, P., Poirier, G. G., and Hannun, Y. A. (1997) *J. Biol. Chem.* **272,** 18530–18533
22. Thornberry, N. A., and Lazebnik, Y. (1998) *Science* **281,** 1312–1316
23. Spector, M. S., Auer, K. L., Jarvis, W. D., Ishac, E. J., Gao, B., Kunos, G., and Dent, P. (1997) *Mol. Cell. Biol.* **17,** 3556–3565
24. Montrose-Rafizadeh, C., Avdonin, P., Garant, M. J., Rodgers, B. D., Kole, S., Yang, H., Levine, M. A., Schwindinger, W., and Bernier, M. (1999) *Endocrinology* **140,** 1132–1140
25. Buteau, J., Roduit, R., Susini, S., and Prentki, M. (1999) *Diabetologia* **42,** 856–864
26. Yao, R., and Cooper, G. M. (1995) *Science* **267,** 2003–2006
27. Thangaraju, M., Sharma, K., Liu, D., Shen, S. H., and Srikant, C. B. (1999) *Cancer Res.* **59,** 1649–1654
28. Sharma, K., Patel, Y. C., and Srikant, C. B. (1999) *Mol. Endocrinol.* **13,** 82–90
29. Turner, P. R., Mefford, S., Christakos, S., and Nissenson, R. A. (2000) *Mol. Endocrinol.* **14,** 241–254
30. Campard, P. K., Crochemore, C., Rene, F., Monnier, D., Koch, B., and Loeffler, J. P. (1997) *DNA Cell Biol.* **16,** 323–333
31. Gu, C., Ma, Y.-C., Benjamin, J., Littman, D., Chao, M. V., and Huang, X.-Y. (2000) *J. Biol. Chem.* **275,** 20726–20733
32. Iwai-Kanai, E., Hasegawa, K., Araki, M., Kakita, T., Morimoto, T., and Sasayama, S. (1999) *Circulation* **100,** 305–311
33. Communal, C., Singh, K., Pimentel, D. R., and Colucci, W. S. (1998) *Circulation* **98,** 1329–1334
34. Communal, C., Colucci, W. S., and Singh, K. (2000) *J. Biol. Chem.* **275,** 19395–19400.
35. Jelinek, L. J., Lok, S., Rosenberg, G. B., Smith, R. A., Grant, F. J., Biggs, S., Bensch, P. A., Kuijper, J. L., Sheppard, P. O., Sprecher, C. A., O'Hara, P. J., Foster, D., Walker, K. M., Chen, L. H. J., McKernan, P. A., and Kindsvogel, W. (1993) *Science* **259,** 1614–1616
36. Drucker, D. J., Philippe, J., Mojsov, S., Chick, W. L., and Habener, J. F. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 3434–3438
37. Mabrouk, G. M., Jois, M., and Brosnan, J. T. (1998) *Biochem. J.* **330,** 759–763
38. Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., and Ullrich, A. (1999) *Nature* **402,** 884–888
39. Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. (1995) *Science* **270,** 1326–1331
40. Li, J., Yang, S., and Billiar, T. R. (2000) *J. Biol. Chem.* **275,** 13026–13034
41. Scaffidi, C., Schmitz, I., Zha, J., Korsmeyer, S. J., Krammer, P. H., and Peter, M. E. (1999) *J. Biol. Chem.* **274,** 22532–22538
42. Tang, D., Lahti, J. M., Grenet, J., and Kidd, V. J. (1999) *J. Biol. Chem.* **274,** 7245–7252
43. Chance, W. T., Foley-Nelson, T., Thomas, I., and Balasubramaniam, A. (1997) *Am. J. Physiol.* **273,** G559–G563