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*

Received for publication, February 9, 2002, and in revised form, April 9, 2002 Published, JBC Papers in Press, April 26, 2002, DOI 10.1074/jbc.M201358200

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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, p90RSK, 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 Ser21 in GSK-3α and at Ser9 in GSK-3β in a PKA-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 Ser155 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), 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 ducal 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 heterogeneous 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-

* This work was supported in part by grants from the Canadian Institutes for Health Research, the National Cancer Institute of Canada, and the Ontario Research and Development Challenge Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a National Science and Engineering Research Council of Canada studentship award.
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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, phosphor (BMA, protease inhibitor mixture (P-5714), and phosphatase inhibitor mixture I were purchased from Sigma. Recombinant human [Gly2]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 benzoylformylarponyl-VAID-fluoromethyl ketone and the kinase inhibitors H89 and LY294002 were obtained from Calbiochem. All electrophoresis reagents were purchased from Bio-Rad. The rabbit polyclonal anti-p90Rsk antibody was purchased 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 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 Ser473, a marker of PI3K activation (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 benzoylformylarponyl-VAID-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 all conditions. GRF (1–29NH2)-containing and -depleted medium (Dulbecco’s 70% confluence, the cultures were maintained for 15–17 h before addition of the drugs. Dimethyl sulfoxide final concentration was identical in all conditions.

**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. 1A). The inhibitory effect of GLP-2 on LY294002-induced cell death was mimicked by forskolin (Fig. 1A), 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. 1A). However, treatment of cells with either GLP-2 or forskolin significantly reduced cell death induced by these agents (Fig. 1A), 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 of GLP-2 or forskolin, cell viability was determined and compared to the viability of untreated controls. As shown in Fig. 2A, GLP-2 and forskolin both significantly inhibited cell death associated with LY294002 treatment when added simultaneously at 6.5 h before analysis. However, when GLP-2 or forskolin were added at 12 h before analysis, cell viability was not further reduced compared to that of LY294002-treated cells. These results suggest that GLP-2R activation is required at 6.5 h prior to analysis to minimize cell death associated with LY294002 treatment. Furthermore, the effectiveness of GLP-2R activation in inhibiting cell death associated with LY294002 treatment was dose-dependent, with maximal protection achieved at 100 nM GLP-2R activation.
of LY294002 as shown in Fig. 1B. Both GLP-2 and forskolin significantly enhanced cell viability at all time points examined (Fig. 1B).

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 Thr308 and Ser473 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 Ser473 phosphorylation from 5 to 360 min following exposure to serum (Fig. 1C). In contrast, co-administration of LY294002 completely abrogated the serum-stimulated phosphorylation of Akt Ser473 during the entire time course of the experiment (Fig. 1C). 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. 1C).

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 benzylxoycarbonyl-VAD-fluoromethyl ketone (Fig. 2A). 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. 2B). 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. 2C). 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(T202/Y204)Erk1/2) as described under "Experimental Procedures." Anti-1xβα antibody was used to monitor loading and transfer conditions. Results are representative of four 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
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. 1C). However, cAMP and PKA have been shown to induce a PI3K-independent activation of Akt that is dependent on Thr308 phosphorylation, but not Ser473 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 Ser473 and Thr308. 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 Akt 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 Thr389 antibody, as the activity of p70 S6 kinase 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 Ser21 and Ser9, 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-3 in BHK-rGLP-2R cells, and GLP-2 treatment further increased GSK-3 phosphorylation (20). Although insulin activated Thr389 phosphorylation of p70 S6 kinase in BHK-rGLP-2R cells, no increase in Thr389 phosphorylation was detected following exposure of cells to forskolin or GLP-2 (Fig. 5). Furthermore, LY294002 completely abolished p70 S6 kinase Thr389 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 p90Rsk, a ribosomal kinase that exerts anti-apoptotic effects on downstream effectors such as Bad (21, 22). Although phorbol esters stimulated phosphorylation of p90Rsk at Ser467, which is critical for the activation of this kinase (23), GLP-2 or forskolin had no effect on p90Rsk Ser467 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 Ser21 in GSK-3α and at Ser9 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 Ser473 phosphorylation, which was mirrored by a comparably rapid reduction in the levels of catalytically inactive GSK-3α and GSK-3β phosphorylated at Ser21 and Ser9, 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 Ser21 and Ser9, 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-2/R signaling to GSK-3 phosphorylation independently of PI3K/Akt, p90Rsk, 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...

![Figure 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. 1A 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 MrR(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).](image-url)
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, p90Rsk, 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 p90Rsk (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. 10A). 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. 10B).
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 Ser21/9 (P(S21/9)GSK-3α/β) antibody was used to detect GSK-3α phosphorylation at Ser21. GSK-3β phosphorylation was examined using anti-phospho-GSK-3β Ser9 (P(S9)GSK-3β) antibody, which demonstrated higher sensitivity than anti-phospho-GSK-3α/β Ser21/9 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.
of Bad at Ser\textsuperscript{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. 2C). 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 directly 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 \(\alpha\)-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

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 \(\pm\) 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\textsuperscript{155} (but not at Ser\textsuperscript{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 \(\mu\)M forskolin for 20 min. Total cell extracts were then analyzed by immunoblotting for phosphorylated Bad (P(S112)Bad and P(S155)Bad), phospho-p90rsk Ser\textsuperscript{380} (P(S380)p90Rsk), and phospho-Akt Ser\textsuperscript{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\textsuperscript{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
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 Ser155 (P(S155)Bad) in a PI3K-independent manner. Inhibition of GSK-3 activity via phosphorylation at Ser21 in GSK-3/ and at Ser9 in GSK-3/ 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 factor, and other cytokines and growth factors can activate a variety of receptor kinases, including the GLP-2R, to trigger survival responses in islet cells.
hormone, vasoactive intestinal peptide, opiates, pituitary adenylyl 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 Gαs-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 adenylyl cyclase-activating polypeptide down-regulate Fas ligand transcription through the type 2 vasoactive intestinal peptide receptor in a CAMP-dependent manner (56). Pituitary adenylyl 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 β1- or β2-adrenergic receptors increases contractility via Gq-dependent coupling to adenylate cyclase, activation of β2-adrenergic receptors inhibits apoptosis via a Gq-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 enhances cell survival 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.

**REFERENCES**

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