

# GLP-1 receptor activation improves $\beta$ cell function and survival following induction of endoplasmic reticulum stress

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## Summary

**Perturbation of endoplasmic reticulum (ER) homeostasis impairs insulin biosynthesis,  $\beta$  cell survival, and glucose homeostasis. We show that a murine model of diabetes is associated with the development of ER stress in  $\beta$  cells and that treatment with the GLP-1R agonist exendin-4 significantly reduced biochemical markers of islet ER stress in vivo. Exendin-4 attenuated translational downregulation of insulin and improved cell survival in purified rat  $\beta$  cells and in INS-1 cells following induction of ER stress in vitro. GLP-1R agonists significantly potentiated the induction of ATF-4 by ER stress and accelerated recovery from ER stress-mediated translational repression in INS-1  $\beta$  cells in a PKA-dependent manner. The effects of exendin-4 on the induction of ATF-4 were mediated via enhancement of ER stress-stimulated ATF-4 translation. Moreover, exendin-4 reduced ER stress-associated  $\beta$  cell death in a PKA-dependent manner. These findings demonstrate that GLP-1R signaling directly modulates the ER stress response leading to promotion of  $\beta$  cell adaptation and survival.**

## Introduction

Glucose homeostasis is preserved through interdependent maintenance of insulin action in peripheral tissues such as liver and muscle, together with basal and nutrient-dependent regulation of insulin secretion from islet  $\beta$  cells. Impairment of insulin action in peripheral tissues is viewed as an early defect contributing to the development of diabetes in susceptible individuals (Kimmerling et al., 1976; Rothman et al., 1995). Although the mechanisms responsible for the development of insulin resistance remain unclear, genetic defects, inflammation, elevated levels of free fatty acids, and impairment of mitochondrial function have been invoked as important determinants of insulin sensitivity in populations at risk for the development of diabetes (Lowell and Shulman, 2005; Perseghin et al., 2003). Intriguingly, many of the molecular and metabolic defects contributing to the development of insulin resistance are also implicated in the pathogenesis of impaired  $\beta$  cell function and loss of  $\beta$  cell mass that accompany the loss of glucose control in diabetic subjects (Rhodes, 2005).

The requirement for enhanced production and secretion of insulin in the setting of increasing insulin resistance necessitates that the diabetic  $\beta$  cell adapt by upregulating insulin biosynthesis while simultaneously preserving  $\beta$  cell survival. Paradoxically, inadequate control of glycemia has been associated with activation of both oxidative and endoplasmic reticulum (ER) stress signaling pathways (Kaneto et al., 2005; Kharroubi et al., 2004; Laybutt et al., 2002; Robertson, 2004; Wang et al., 2005), leading to reduced insulin secretion and enhanced susceptibility to  $\beta$  cell death. Furthermore, insulin secretagogues of the sulphonylurea class robustly increase insulin exocytosis, but not insulin biosynthesis (Garcia et al., 1976), and may be associated with induction of  $\beta$  cell apoptosis in vitro (Efanova et al., 1998; Maedler et al., 2005).

Several independent lines of genetic evidence implicate a role for ER homeostasis in  $\beta$  cell function and survival. For example, mutations in the insulin gene resulting in misfolding of insulin, and both enlargement and dysfunction of the ER, lead to reduced  $\beta$  cell insulin secretion and diabetes (Wang et al., 1999a). Similarly, mutations in key components of the ER stress response also lead to loss of  $\beta$  cell mass and diabetes in rodents (Harding et al., 2001; Scheuner et al., 2001). Furthermore, components of the ER stress pathway are also essential for  $\beta$  cell function in human subjects, as exemplified by the development of  $\beta$  cell loss and diabetes in individuals harboring mutations in the pancreatic ER kinase (PERK) (Delepine et al., 2000) and in the WFS1 gene (Fonseca et al., 2005; Inoue et al., 1998).

The signaling pathways engaged following ER stress are complex and involve three different arms of the unfolded protein response (UPR) (Ron, 2002; Wek et al., 2006; Wu and Kaufman, 2006) whose activation relies on the ER stress luminal sensors ATF6, IRE1, and PERK. Activated PERK phosphorylates the translation initiation factor eIF2 $\alpha$ , which transiently attenuates global protein synthesis, reducing the load on the ER. A second arm of the UPR, mediated by activated IRE1, splices XBP-1 mRNA leading to generation of an active transcription factor. Finally, intramembrane cleavage releases the cytosolic domain of ATF6 that also functions as a transcription factor. Both XBP-1 and ATF6 are involved in the activation of genes encoding molecular chaperones and proteins involved in ER-associated degradation and are therefore important to enhance ER function. ER stress-mediated eIF2 $\alpha$  phosphorylation and ensuing repression of general translation allow the dysfunctional ER a window of opportunity to recover from cellular stress. However, eIF2 $\alpha$  phosphorylation also leads to enhanced translation of ATF-4 (Harding et al., 2000a; Lu et al., 2004; Vattem and Wek, 2004), which in turn upregulates the expression of key genes important

for the recovery from ER stress. Translational recovery in ER-stressed cells is mediated by GADD34, a regulatory protein that recruits and activates the catalytic subunit of type 1 protein phosphatase (PP1c), targeting it to the ER to promote eIF2 $\alpha$  dephosphorylation (Ma and Hendershot, 2003; Novoa et al., 2001; Novoa et al., 2003).

A growing body of evidence suggests that incretin hormones, exemplified by glucagon-like peptide-1 (GLP-1), not only increase insulin secretion but also act directly on the  $\beta$  cell to upregulate insulin biosynthesis, while enhancing resistance to  $\beta$  cell death (Buteau et al., 2004; Farilla et al., 2003; Jhala et al., 2003; Li et al., 2003; Perfetti et al., 2000; Wang et al., 2004). To explore potential mechanisms coupling GLP-1 receptor (GLP-1R) activation to enhanced insulin translation and the prevention of  $\beta$  cell apoptosis, we searched for a connection between G protein-coupled receptor (GPCR)-activated signaling pathways linking GLP-1 action to modulation of ER stress. We show here that engagement of the GLP-1R directly regulates the PERK arm of the UPR, providing a mechanism for  $\beta$  cell adaptation to metabolic and cellular stress.

## Results

GLP-1R agonists have previously been shown to either prevent or ameliorate experimental diabetes and preserve  $\beta$  cell mass in multiple preclinical models (Drucker, 2006), including the db/db mouse (Kim et al., 2003; Wang and Brubaker, 2002). Accordingly, we examined glucose control, islet histology, and the expression of markers important for the response to ER stress in diabetic mice treated with vehicle alone or with exendin-4, a potent GLP-1R agonist. The transcription factor CHOP subserves a number of important cellular functions and has also been implicated as a critical component of the ER stress response (Oyadomari and Mori, 2004). We detected a progressive increase in CHOP-positive nuclei following serial analysis of islets from db/db mice in the absence of exendin-4 treatment at various ages (data not shown). Furthermore, treatment of db/db mice with exendin-4 for 3 weeks produced a significant decrease in CHOP expression in islet  $\beta$  cells (Figure 1A), together with a reduction in levels of blood glucose (Figure 1B), and a significant increase in levels of mRNA transcripts for insulin and IRS-2 (Figure 1C). Furthermore, exendin-4 markedly reduced the pancreatic levels of spliced XBP-1 (sXBP-1) RNA transcripts in the same experiment (Figure 1D). Taken together, these data illustrate that the GLP-1R agonist exendin-4 reduces pancreatic expression of CHOP and sXBP-1, molecular mediators involved in the cellular response to ER stress.

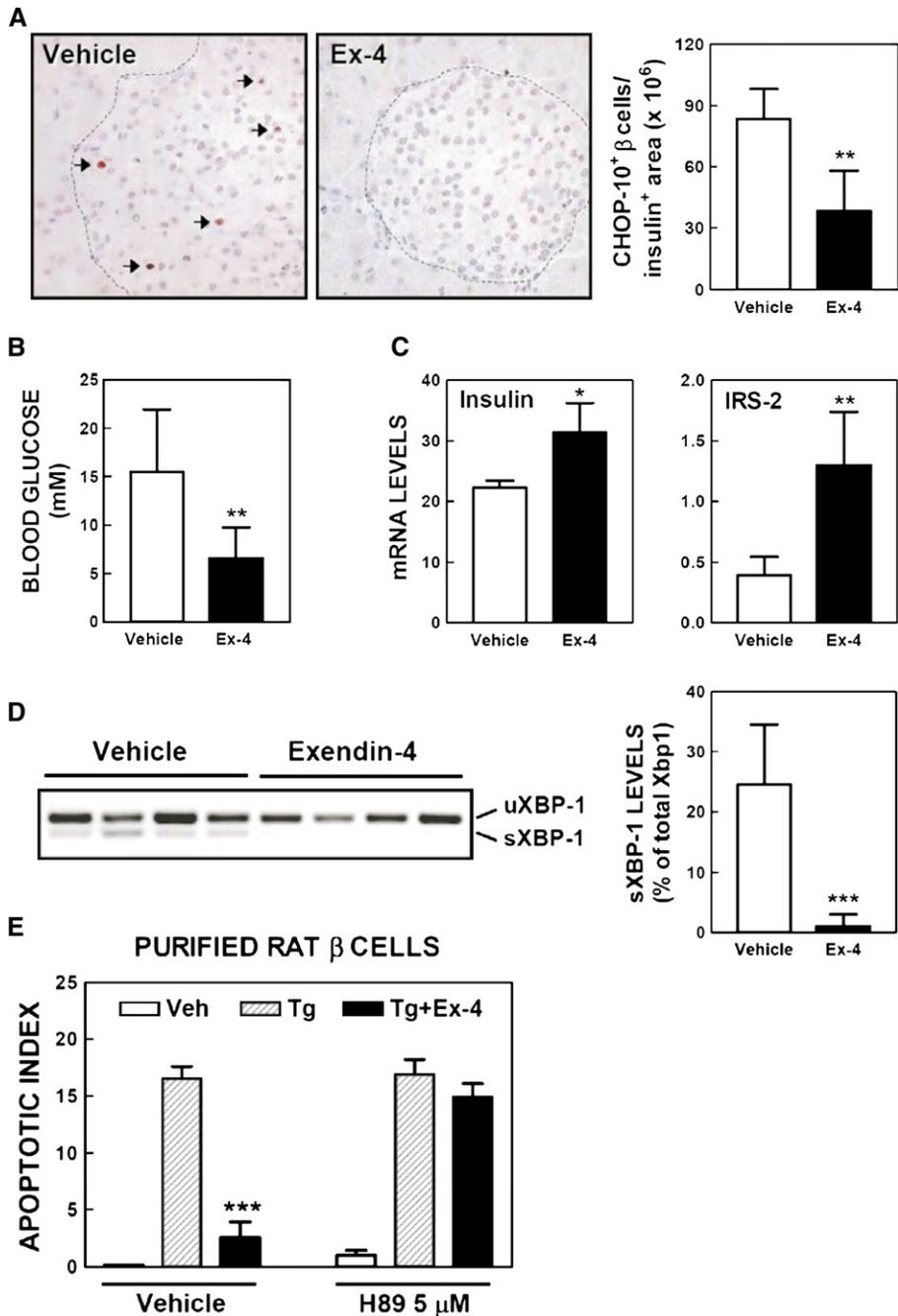
To ascertain whether GLP-1R activation directly regulates the ER stress response in  $\beta$  cells, we treated cultured purified rat  $\beta$  cells with thapsigargin, a pharmacological inducer of ER stress, for 12 hr in the presence or absence of exendin-4 or forskolin. Exendin-4 and forskolin significantly suppressed thapsigargin-induced  $\beta$  cell apoptosis in isolated rat  $\beta$  cells (Figure 1E and data not shown). Furthermore, the cytoprotective actions of exendin-4 were completely abrogated by H89, a protein kinase A inhibitor (Figure 1E). Similarly, thapsigargin markedly suppressed insulin synthesis in short-term cultures of isolated rat  $\beta$  cells (Table 1), whereas exendin-4 and forskolin (Table 1) upregulated insulin synthesis in thapsigargin-treated rat  $\beta$  cells in vitro. Hence, these findings illustrate that GLP-1R activation attenuates the expression of CHOP and sXBP-1 in  $\beta$  cells of dia-

betic mice and directly engages pathways modulating ER stress-associated translational repression and cell death in isolated nontransformed  $\beta$  cells.

To determine whether GLP-1R signaling also regulated components of the UPR in islet cell lines, we exposed rat insulinoma INS-1(832/13) cells to pharmacological inducers of ER stress in the presence or absence of exendin-4. Development of the ER stress response was assessed by measuring the levels of ATF-4 and sXBP-1, reporters of activation of the PERK and IRE1 arms, respectively, of the UPR (Harding et al., 2002; Kaufman et al., 2002; Shen et al., 2004). Both thapsigargin and tunicamycin upregulated ATF-4 and sXBP-1 protein levels in INS-1(832/13) cells, albeit with different kinetics (Figure 2A). Furthermore, exendin-4 markedly enhanced the magnitude of ATF-4 and sXBP-1 induction in the presence of either thapsigargin or tunicamycin (Figure 2A). Additional experiments demonstrated that the effects of exendin-4 on sXBP-1 in ER-stressed INS-1 cells are associated with increased expression of chaperone-encoding genes such as *DnaJb9* (see Figure S1 in the Supplemental data available with this article online). The upregulation of ATF-4 and sXBP-1 following ER stress was also potentiated by exendin-4 in the parental INS-1 cell line (data not shown) and in the mouse insulinoma cell line MIN6 (Figure 2B). These results demonstrate that activation of GLP-1R signaling modifies the UPR in  $\beta$  cell lines via direct upregulation of ATF-4 and sXBP-1 expression.

As ATF-4 is translationally induced during ER stress in response to PERK-mediated eIF2 $\alpha$  phosphorylation (Harding et al., 2000a), we examined whether exendin-4 regulates ATF-4 induction via modulation of eIF2 $\alpha$  phosphorylation. The exendin-4-mediated overinduction of ATF-4 expression was associated with decreased levels of phospho-Ser51 eIF2 $\alpha$  compared to the levels detected in cells treated with thapsigargin or tunicamycin alone (Figure 2C). Moreover, exendin-4 increased the levels of CHOP mRNA transcripts (Figure S2) and CHOP protein (Figure 2C), a target of ATF-4 transcriptional regulation (Harding et al., 2000a; Jiang et al., 2004; Ma et al., 2002), following incubation of INS-1 cells with either thapsigargin or tunicamycin. These observations demonstrate that exendin-4 regulates not only ATF-4 but also enhances the expression of downstream targets of ATF-4. Furthermore, the effects of exendin-4 on eIF2 $\alpha$  phosphorylation and both ATF-4 and CHOP induction were reproduced with native GLP-1 (Figure S3A), the endogenous ligand for the mammalian GLP-1R, with the GIP receptor agonist [D-Ala<sup>2</sup>]GIP (Figure S3B) and with forskolin (Figure 2C and Figure S3A), implicating cAMP as a potential intermediary linking incretin hormone receptor activation to the PERK arm of the ER stress response.

Activation of the GLP-1R in  $\beta$  cells engages cAMP/PKA, Erk1/2 MAP kinase, and PI-3 kinase/Akt signaling (Buteau et al., 1999, 2001; Drucker, 2006; Drucker et al., 1987; Wang and Brubaker, 2002). To identify mechanisms coupling GLP-1R activation to the modulation of the ER stress response, we used kinase inhibitors to selectively inactivate intracellular signaling pathways in the presence or absence of exendin-4. The effectiveness of the different inhibitors to block the activity of their corresponding target kinases in INS-1 cells is illustrated in Figure S6. Incubation of INS-1 cells with either H89, U0126, or LY294002 alone did not interfere with the activation of the PERK arm of the UPR in response to ER stress (Figure 2D). However, the PKA inhibitor H89 prevented the potentiation of ATF-4 and CHOP induction



**Figure 1.** Exendin-4 reduces the expression of markers of ER stress in  $\beta$  cells from db/db mice and enhances survival of purified rat  $\beta$  cells subjected to ER stress

db/db mice (4 weeks old) were given twice-daily i.p. injections of PBS (vehicle) or exendin-4 (Ex-4) for 3 weeks. Body weights at the end of the 3-week treatment period were  $30.9 \pm 0.8$  g versus  $28.6 \pm 0.7$  g for PBS versus Ex-4-treated mice,  $p < 0.05$ . Blood glucose levels were determined and pancreata were obtained for immunohistochemical and RNA analyses.

**A)** Photomicrographs of representative islet CHOP staining (arrows point to CHOP-positive nuclei) in vehicle- and exendin-4-treated mice. Islet perimeter is marked by a dashed line. Magnification, 400 $\times$ . The number of CHOP-positive  $\beta$  cells normalized per  $\beta$  cell area was quantified as described in [Experimental Procedures](#). Data are means  $\pm$  SD ( $n = 6$  mice per group). Analysis of serial adjacent sections stained with antisera to insulin and CHOP demonstrated that the majority of CHOP-positive islet cells corresponded to  $\beta$  cells;  $80.3\% \pm 14.4\%$  (saline-treated mice) and  $88.4\% \pm 12\%$  (exendin-4-treated mice). CHOP-positive nuclei were rarely seen in exocrine pancreas.

**B)** Blood glucose levels in vehicle- and exendin-4-treated mice immediately prior to sacrifice. Data are means  $\pm$  SD ( $n = 9$  mice per group).

**C)** Pancreatic insulin and IRS-2 mRNA levels assessed by real-time quantitative RT-PCR in vehicle- and exendin-4-treated mice. Data are means  $\pm$  SD ( $n = 4$  mice per group) and are normalized to levels of glucokinase mRNA transcripts in the same RNA preparations.

**D)** PCR products amplified from unspliced (uXBP-1) and spliced (sXBP-1) pancreatic XBP-1 mRNA from four vehicle- and four exendin-4-treated mice. The percentage of sXBP-1 to total XBP-1 determined by densitometry is illustrated. Data are means  $\pm$  SD ( $n = 4$  mice per group). For **(A)–(D)**, \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  for Ex-4 versus vehicle.

**E)** Purified rat  $\beta$  cells were exposed to either vehicle alone or H89 during a 12 hr treatment with vehicle (Veh) or 0.5  $\mu$ M thapsigargin (Tg) in the absence or presence of 10 nM exendin-4 (Ex-4). The apoptotic index was calculated as described in [Experimental Procedures](#). Data represent means  $\pm$  SE of four independent experiments. \*\*\* $p < 0.001$  for Tg+Ex-4 versus Tg alone.

and the attenuation of eIF2 $\alpha$  phosphorylation by exendin-4 or forskolin in thapsigargin-treated cells ([Figure 2D](#)). In contrast, the MEK1/2 inhibitor U0126 or the PI-3 kinase inhibitor LY294002 had no effect on exendin-4-mediated changes in ATF-4, CHOP, or eIF2 $\alpha$  phosphorylation ([Figure 2D](#)). Identical results were obtained using PD98059 and wortmannin, different inhibitors for MEK1/2 and PI-3 kinase, respectively (data not shown). These observations strongly suggest that cAMP and PKA are the intracellular mediators involved in the modulation of the PERK arm of the UPR by GLP-1R agonists.

To explore the mechanisms responsible for the paradoxical relationship between reduced eIF2 $\alpha$  phosphorylation yet increased ATF-4 levels in INS-1 cells undergoing ER stress in

the presence of GLP-1R agonists or forskolin, we performed a detailed analysis of the temporal relationship between ATF-4 induction and eIF2 $\alpha$  phosphorylation. Levels of ATF-4 were increased by 1 hr of thapsigargin treatment with maximal induction occurring following 4–6 hr of exposure ([Figure 3A](#); see also [Figure 2A](#) and [Figure 4C](#)). Forskolin did not modify the temporal profile of ATF-4 accumulation but significantly increased the magnitude of ATF-4 induction. Similarly, CHOP was also induced by thapsigargin and further upregulated by forskolin yet with delayed kinetics compared to ATF-4 ([Figure 3A](#)), consistent with ATF-4's role as an upstream activator of the CHOP promoter ([Harding et al., 2000a; Jiang et al., 2004; Ma et al., 2002](#)). Phosphorylation of eIF2 $\alpha$  increased rapidly in

**Table 1.** Effects of exendin-4 and forskolin on thapsigargin-induced suppression of insulin synthesis in rat pancreatic  $\beta$  cells

Conditions		Insulin synthesis (d.p.m./cell $\times$ hr)	
Tg	Ex-4/Fk	0 mM glucose	10 mM glucose
Experiment 1 (n = 4)			
-	-	0.20 $\pm$ 0.06	3.00 $\pm$ 0.27
-	Ex-4	0.36 $\pm$ 0.11	3.23 $\pm$ 0.34
+	-	0.04 $\pm$ 0.01	0.90 $\pm$ 0.09
+	Ex-4	0.11 $\pm$ 0.02 <sup>a</sup>	1.21 $\pm$ 0.07 <sup>b</sup>
Experiment 2 (n = 6)			
-	-	0.27 $\pm$ 0.04	3.30 $\pm$ 0.37
-	Fk	1.24 $\pm$ 0.23 <sup>a</sup>	4.12 $\pm$ 0.51
+	-	0.07 $\pm$ 0.02	1.22 $\pm$ 0.16
+	Fk	0.42 $\pm$ 0.08 <sup>a</sup>	2.11 $\pm$ 0.30 <sup>a</sup>

Rat  $\beta$  cells were cultured for 8 hr with or without thapsigargin (Tg, 0.5  $\mu$ M) in the presence of exendin-4 (Ex-4, 10 nM) or forskolin (Fk, 20  $\mu$ M). Data represent mean  $\pm$  SE of (n) independent experiments.

<sup>a</sup>  $p < 0.01$ , <sup>b</sup>  $p < 0.05$  versus control cultures in the absence of Ex-4 or Fk.

thapsigargin-treated INS-1 cells and was sustained for 4 hr (Figure 3B). Coincubation of INS-1 cells with thapsigargin and either exendin-4 or forskolin did not affect the initial phase of eIF2 $\alpha$  phosphorylation kinetics; however, exendin-4 or forskolin reduced the levels of eIF2 $\alpha$  phosphorylation from 2 to 4 hr after thapsigargin exposure (Figure 3B). Hence, overinduction of ATF-4 expression by exendin-4 and/or forskolin during ER stress is an early event relative to the subsequent decrease in eIF2 $\alpha$  phosphorylation, raising the possibility that activation of GLP-1R signaling in  $\beta$  cells undergoing ER stress results in modulation of ATF-4 induction and eIF2 $\alpha$  phosphorylation by different mechanisms.

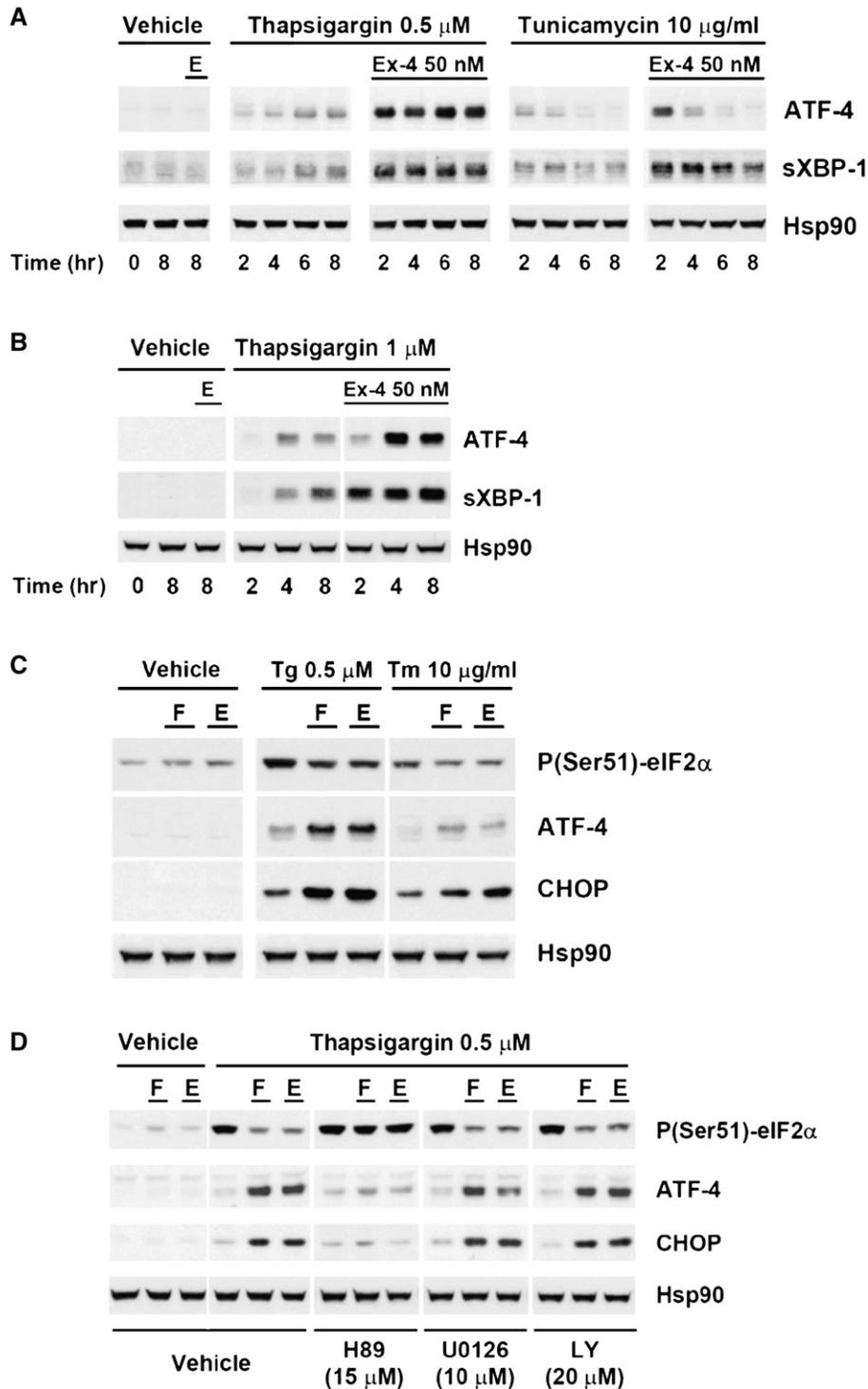
PERK is the primary eIF2 $\alpha$  kinase induced by ER stress (Harding et al., 1999; Jiang et al., 2004). To ascertain its contribution to the decrease in phospho-eIF2 $\alpha$  in ER-stressed  $\beta$  cells treated with GLP-1R agonists, we assessed the activation state of PERK by following the characteristic shift in mobility of the phosphorylated active form of the protein by SDS-PAGE (Harding et al., 1999, 2000a). PERK phosphorylation was detectable in INS-1 cells as early as 20 min following thapsigargin treatment, was maintained for at least 4 hr, and was not prevented but was actually accentuated by coincubation with exendin-4 or forskolin (Figure 3C). As a reduction in eIF2 $\alpha$  phosphorylation was prominent in exendin-4-treated cells (Figure 3B) despite persistent activation of the upstream kinase PERK (Figure 3C), these findings demonstrate that exendin-4 exerts its effects on eIF2 $\alpha$  phosphorylation downstream of PERK.

To determine the functional significance of the changes in phospho-eIF2 $\alpha$  levels in ER-stressed  $\beta$  cells treated with GLP-1R agonists, we measured the incorporation of radiolabeled leucine into newly synthesized proteins. Phosphorylation of the  $\alpha$  subunit of eIF2 attenuates eIF2-GTP-tRNA<sup>met</sup> ternary complex formation, thereby reducing global translation initiation and polypeptide biosynthesis, which is followed by a phase of eIF2 $\alpha$  dephosphorylation and translational recovery (Harding et al., 2002; Kaufman et al., 2002). Thapsigargin produced a rapid and profound repression of translation in INS-1 cells, followed by a slow and quantitatively modest recovery after several hours (Figure 4A). Exendin-4 markedly enhanced the magnitude of translational recovery following thapsigargin ex-

posure (Figure 4A). The effect of exendin-4 on recovery from ER stress-induced translational repression was mimicked by forskolin (Figures 4A and 4B) and abrogated by preincubation with H89 (Figure 4B). Although H89 attenuated global protein synthesis on its own, this effect did not seem to result from cytotoxicity. Glucose-stimulated protein synthesis is brought about mainly by dephosphorylation of eIF2 $\alpha$  in  $\beta$  cells (Gomez et al., 2004) and was preserved following exposure of INS-1 cells to H89 either in the absence or presence of concomitant ER stress (data not shown). These results indicate that exendin-4 acting downstream of PERK attenuates phospho-eIF2 $\alpha$  levels, thus promoting a faster resumption of protein synthesis during ER stress.

Dephosphorylation of eIF2 $\alpha$  is catalyzed by the ER stress-inducible gene *Gadd34*. *Gadd34* encodes a regulatory protein that recruits and activates the catalytic subunit of PP1c, targeting it to the ER to promote eIF2 $\alpha$  dephosphorylation and translational recovery from the early phase of translational repression during the ER stress response (Novoa et al., 2001, 2003). ATF-4 (Jiang et al., 2004; Ma and Hendershot, 2003) and CHOP (Marciniak et al., 2004) directly activate *Gadd34*, delineating a negative feedback loop that controls protein translation during ER stress. To ascertain whether GLP-1R signaling engages GADD34, resulting in reduced phospho-eIF2 $\alpha$ , we measured the levels of PP1c-associated GADD34, an indicator of the abundance of the ER stress-induced eIF2 $\alpha$  holophosphatase complex. PP1c-associated GADD34 was isolated by microcystin affinity chromatography, which efficiently purifies PP1c owing to its high-capacity binding to this phosphatase (Campos et al., 1996). The GADD34-PP1c complex, but not PP1c alone, was expressed at low levels in unstressed cells. Thapsigargin induced PP1c-associated GADD34 expression, which was further augmented in cells coincubated with exendin-4 or forskolin (Figure 4C). Furthermore, the abundance of the GADD34-PP1c complex correlated with the eIF2 $\alpha$  dephosphorylation kinetics following ER stress in the presence of exendin-4 or forskolin (Figure 4C). Both the overinduction of GADD34 and the associated reduction in levels of phospho-eIF2 $\alpha$  in thapsigargin-treated INS-1 cells coincubated with either exendin-4 or forskolin was prevented by H89 (Figure 4D). Exendin-4 also upregulated the expression of GADD34 mRNA transcripts following induction of ER stress in INS-1 cells treated with tunicamycin (Figure S4). These results indicate that exendin-4, in a cAMP- and PKA-dependent manner, potentiates the induction of the eIF2 $\alpha$  phosphatase complex during the ER stress response, resulting in attenuation of levels of phospho-eIF2 $\alpha$  and a faster recovery from translational repression.

The previous results highlight the functional significance of the pathways regulated by exendin-4 that contribute to relief of ER stress-mediated translational repression in INS-1 cells. To determine how GLP-1 receptor agonists promote induction of ATF-4, we assessed the regulation of ATF-4 mRNA during ER stress. Levels of ATF-4 transcripts were increased following treatment of INS-1 cells with thapsigargin alone; however, they were not further upregulated following coincubation with exendin-4 or forskolin (Figure 5A), suggesting a posttranscriptional mechanism for the actions of exendin-4 on ATF-4 induction during ER stress. This conclusion was further supported by the observation that treatment with actinomycin D did not abrogate the further induction of ATF-4 protein by exendin-4 or forskolin following incubation of INS-1 cells with thapsigargin for



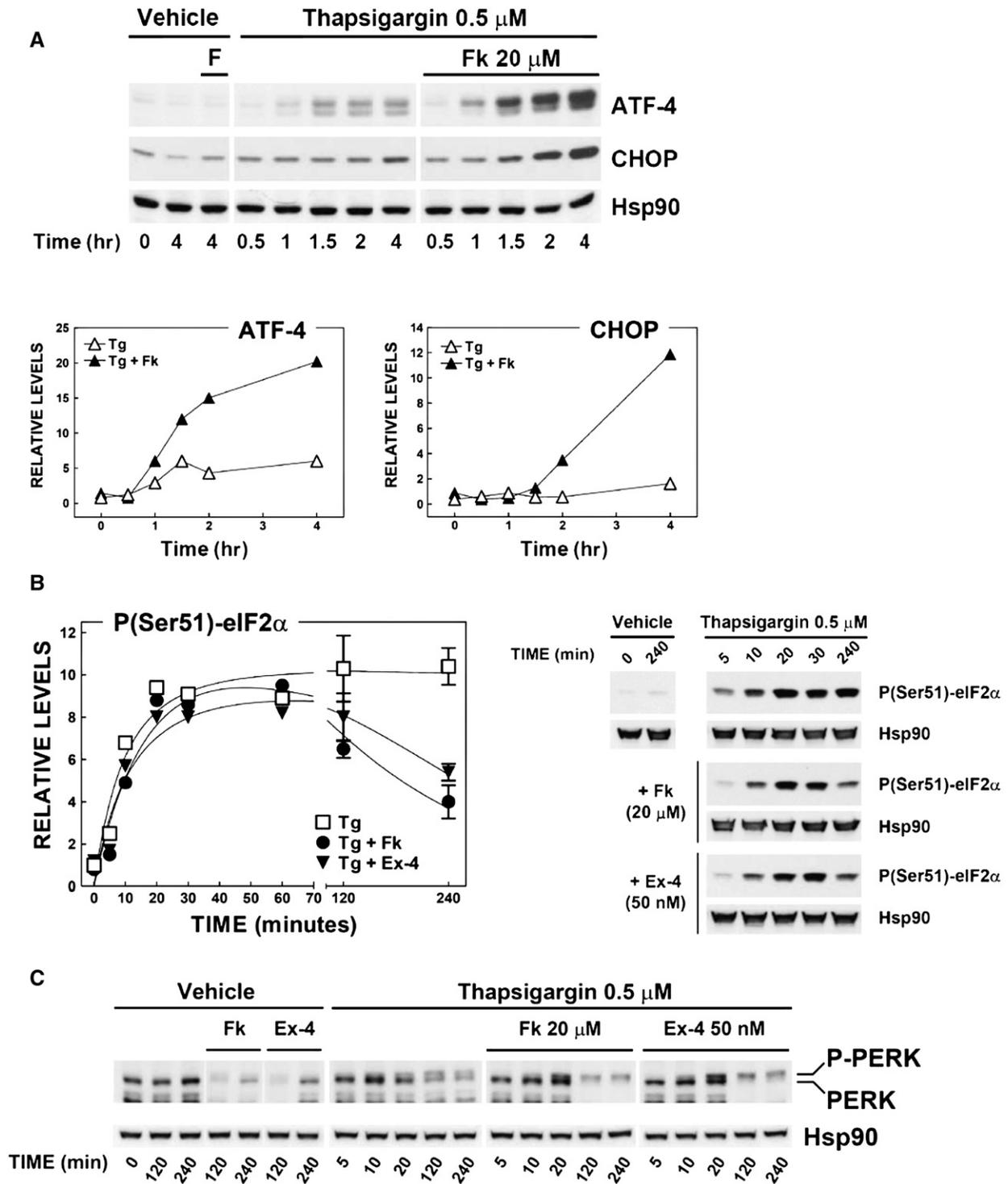
**Figure 2.** Exendin-4 regulates both the PERK and the IRE1/XBP-1 arms of the UPR in rodent insulinoma cell lines

Rat INS-1 (**A**, **C**, and **D**) or mouse MIN6 (**B**) insulinoma cells were treated with vehicle alone, thapsigargin (Tg), or tunicamycin (Tm) in the absence or presence of 50 nM exendin-4 (Ex-4 or E) or 20  $\mu$ M forskolin (Fk or F) for the indicated periods of time (**A** and **B**) or for 4 hr (**C** and **D**). In (**D**), cultures were exposed to vehicle alone or to either H89, U0126, or LY294002 (LY) for 20 min prior to and during the 4 hr treatment with thapsigargin in the absence or presence of exendin-4 or forskolin. Total cell extracts were analyzed by immunoblotting for ATF-4, XBP-1, phospho-eIF2 $\alpha$  Ser51 (P[Ser51]-eIF2 $\alpha$ ), and CHOP as indicated. The signal shown in the XBP-1 immunoblot corresponds to the ~54 kDa protein encoded by the sXBP-1 mRNA (sXBP-1). Anti-Hsp90 antibody was used to monitor loading and transfer conditions. Results shown in (**A**), (**B**), and (**C**) are representative of four, three, and two independent experiments, respectively. In (**D**), results are representative of four (H89) and two (U0126 and LY294002) similar experiments.

1 hr (Figure S5A). To determine whether the effect of exendin-4 on ER stress-triggered ATF-4 induction was mediated by increased protein stability, we examined the rate of ATF-4 decay after translational inhibition with cycloheximide. In contrast to the relative stability of CHOP and Hsp90, levels of ATF-4 protein decayed rapidly following cycloheximide treatment (Figure 5B), consistent with the previously reported instability of ATF-4 (Blais et al., 2004; Lassot et al., 2001). The  $t_{1/2}$  of ATF-4 protein was

~20 min in cells exposed to thapsigargin alone and did not change following coincubation with exendin-4 or forskolin. Hence, ATF-4 protein stabilization does not contribute to the GLP-1R-dependent potentiation of ATF-4 protein accumulation during ER stress.

Previous studies have implicated the 5'UTR of the ATF-4 mRNA in its translational regulation by ER stress-induced eIF2 $\alpha$  phosphorylation (Harding et al., 2000a; Lu et al., 2004;



**Figure 3.** Exendin-4 affects eIF2 $\alpha$  phosphorylation in ER-stressed INS-1 cells downstream of PERK

Cells were exposed to vehicle alone or thapsigargin (Tg) in the absence or presence of exendin-4 (Ex-4) or forskolin (Fk or F) for the indicated periods of time. Total cell extracts were then analyzed by immunoblotting for ATF-4 and CHOP (A), P(Ser51)-eIF2 $\alpha$  (B), PERK (C), and Hsp90 (loading control). Plotted in (A) and (B) are the intensity of the ATF-4 and CHOP signals and of the P(Ser51)-eIF2 $\alpha$  signal, respectively, quantified by densitometry, corrected by the intensity of the Hsp90 signal, and expressed relative to the values at time zero. Data in (A) are means from two independent experiments. Data in (B) are the means  $\pm$  SEM from three to four independent experiments. Coefficients of variation were  $\leq$  15% for the time points in the kinetics whose SEM has been omitted for the purpose of clarity. (C) depicts the active phosphorylated form of PERK (P-PERK) with reduced mobility compared to its inactive underphosphorylated form (PERK). Blots shown in (C) are representative of three independent experiments.

Vattem and Wek, 2004). Therefore, we examined the activity of a transfected plasmid construct carrying the murine ATF-4 mRNA 5'UTR and initiation codon fused to the luciferase reporter gene (TK-ATF4UTR-Luc) (Harding et al., 2000a). Reporter gene activity was unaffected by incubation of INS-1 cells with either exendin-4 or forskolin alone (Figure 5C). However, luciferase expression was increased 4- to 5-fold following exposure of the cells to thapsigargin for 8 hr, and both exendin-4 and forskolin further enhanced luciferase activity by ~8-fold in thapsigargin-treated cells (Figure 5C). Because levels of ATF-4-luciferase mRNA transcripts did not change regardless of the treatment condition (Figure 5C), these results support a translational mechanism for the overinduction of ATF-4 protein expression by exendin-4 during ER stress.

To independently address the same issue using a different experimental technique, we measured the rate of ATF-4 protein synthesis in INS-1 cells by pulse labeling with [<sup>35</sup>S]Met/Cys and immunoprecipitation with an ATF-4-specific antibody. As shown in Figure 5D, incorporation of [<sup>35</sup>S]Met/Cys into nascent ATF-4 followed the same pattern as the changes in levels of ATF-4 protein assessed by western blotting. Incorporation of [<sup>35</sup>S]Met/Cys into ATF-4 was comparatively low in unstressed vehicle-treated cells or in cells treated with exendin-4 alone. In contrast, INS-1 cells exposed to a 1 hr thapsigargin challenge exhibited a 2- to 4-fold increase in newly synthesized ATF-4 and a further increase in newly synthesized ATF-4 (43.0% ± 8.3%, n = 4 independent experiments) was observed in cells treated with both thapsigargin and exendin-4 (Figure 5D). General translation rate, as assessed by the incorporation of [<sup>35</sup>S]Met/Cys into proteins, was inhibited to the same extent following thapsigargin treatment for 1 hr either in the absence or presence of exendin-4, in agreement with the parallel induction of similar eIF2 $\alpha$  phosphorylation levels under both conditions (Figure 5D).

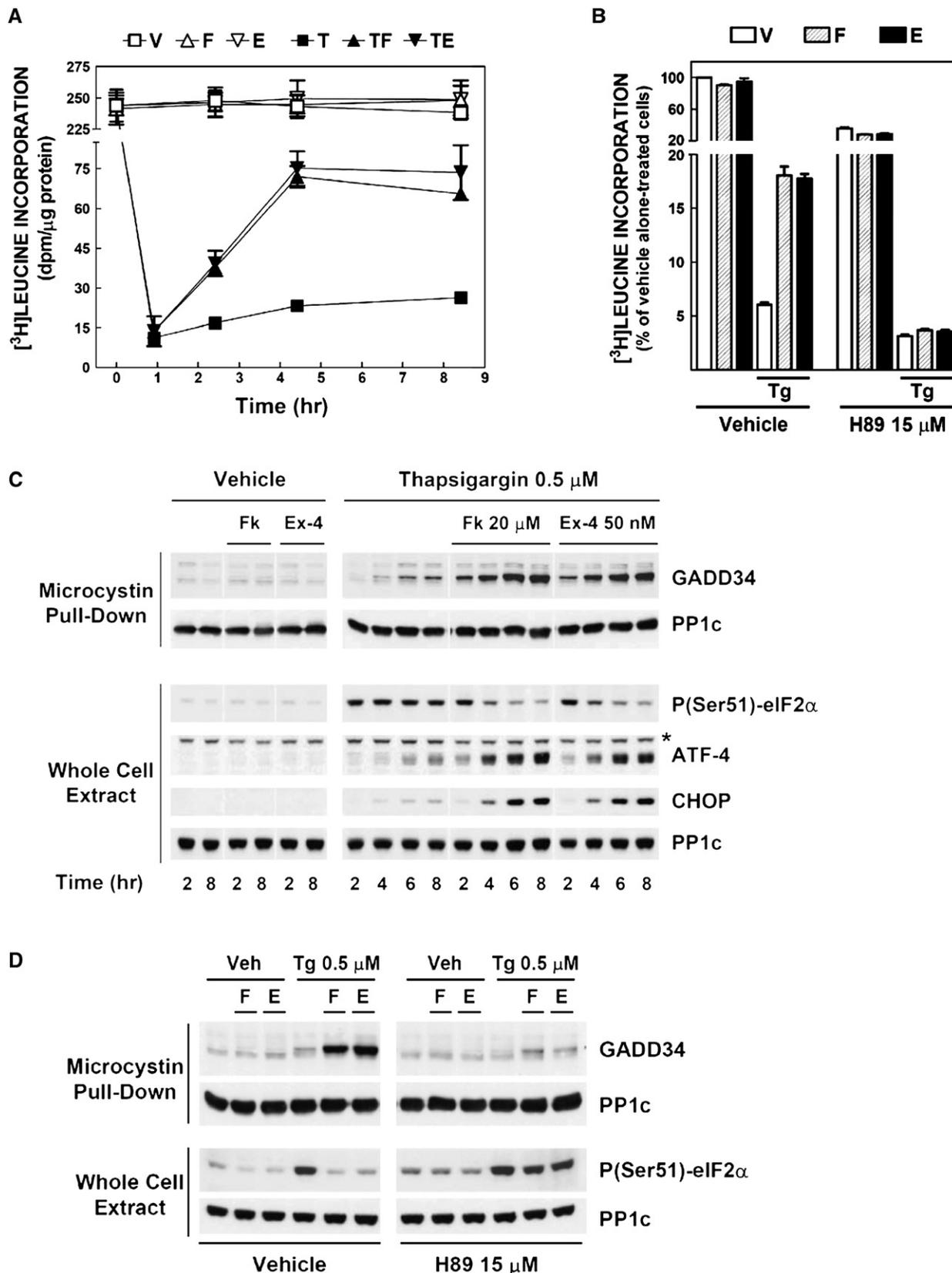
The UPR in mammalian cells promotes adaptation to ER stress imposed by physiological requirements for upregulation of protein synthesis and secretion, leading to maintenance of ER homeostasis and cell survival. Although persistent activation of the UPR may lead to apoptosis, thereby eliminating cells with irreparable damage caused by severe ER stress (Harding et al., 2002; Shen et al., 2004), we observed that exendin-4 improved cell survival following induction of ER stress in primary cultures of rat  $\beta$  cells (Figure 1E). To determine the mechanisms linking GLP-1R activation with enhanced cell survival in the presence of ER stress, INS-1 cells were subjected to a thapsigargin challenge for 8 hr in the presence or absence of exendin-4 or forskolin, followed by recovery in regular growth media. In this experiment, outgrowth of cell colonies after thapsigargin treatment reflects cell survival following induction of ER stress. Consistent with the data depicted in Figure 1E, treatment of INS-1 cells with either exendin-4 or forskolin for 8 hr produced more numerous and larger cell colonies after a concomitant thapsigargin pulse (Figure 6A). GLP-1, exendin-4, and forskolin independently enhanced INS-1 cell colony formation in similar experiments (Figure 6B). As GLP-1R agonists attenuate apoptosis and promote proliferation of pancreatic  $\beta$  cells (Drucker, 2003), we examined the impact of exendin-4 on  $\beta$  cell proliferation and survival in the setting of ER stress. Within 24 hr of the transient thapsigargin pulse, INS-1 cells demonstrated signs of apoptosis, including membrane blebbing, cell detachment, chromatin condensation, and nuclear disintegration and cell fragmentation

into apoptotic bodies (data not shown). The manifestation of these changes preceded a marked reduction in the number of viable cells during the next 48 hr followed by resumption of cell growth (Figure 6C). Coincubation of INS-1 cells with exendin-4 or forskolin during the initial 8 hr exposure to thapsigargin markedly reduced the subsequent loss of viable cells within the first 3 days. However, cell growth resumed and appeared comparable in all three groups at later time points (Figure 6C). Furthermore, exposure to exendin-4 or forskolin alone in the absence of thapsigargin for 8 hr did not affect subsequent INS-1 cell growth (Figure 6D). Consequently, the enhanced cell colony outgrowth observed in INS-1 cell cultures exposed to exendin-4 or forskolin during the thapsigargin pulse is most consistent with inhibitory actions of exendin-4 and forskolin on thapsigargin-induced cell death. Similar results were obtained in identical experiments performed using MIN6 cells (data not shown). Furthermore, stimulation of endogenous insulin release by tolbutamide or glybenclamide, or addition of exogenous insulin, did not affect survival in ER-stressed INS-1 cells. Moreover, inhibition of insulin exocytosis by diazoxide did not abrogate the prosurvival effect of exendin-4 in INS-1 cells undergoing ER stress (data not shown). These results suggest that insulin released following exendin-4 treatment of INS-1 cells does not play a role in the ability of GLP-1R agonists to inhibit ER stress-mediated cell death.

The role of different intracellular signaling pathways in mediating the enhanced survival of exendin-4-treated INS-1 cells following ER stress-induced cell death was assessed using kinase inhibitors. Inhibition of MEK1/2 kinase by PD98059 or U0126 did not attenuate exendin-4 or forskolin-mediated enhancement of INS-1 cell colony outgrowth after a thapsigargin challenge (Figure 6E). Similarly, the PI-3 kinase inhibitors wortmannin or LY294002 failed to diminish the prosurvival actions of exendin-4 or forskolin in the same experiments (Figure 6F). These results indicate that both MEK1/2 kinase and PI-3 kinase are dispensable for the inhibitory effects of exendin-4 and forskolin on ER stress-induced INS-1 cell death. In contrast, H89 significantly attenuated the actions of exendin-4 and forskolin on enhancement of INS-1 cell colony growth (Figure 6G). These observations strongly suggest that cAMP and PKA are key intracellular mediators coupling GLP-1R activation to modulation of ER stress-induced cell death in INS-1 cells.

## Discussion

The observation that human diabetic subjects exhibit an early defect in glucose-stimulated insulin secretion (Bagdade et al., 1967), together with findings from autopsy studies demonstrating reduced  $\beta$  cell mass in diabetic subjects (Butler et al., 2003), have focused efforts on development of intervention strategies that improve  $\beta$  cell function and prevent  $\beta$  cell death. Hence, there is active interest in determining whether newer drugs such as the insulin sensitizing agents will produce sustained improvements in  $\beta$  cell function and thereby alter the natural history of type 2 diabetes (Buchanan et al., 2002; Ovalle and Bell, 2004; Rhodes, 2005). Increasing experimental evidence implicates a role for ER stress in the development of progressive reductions in insulin secretion associated with  $\beta$  cell failure and apoptotic  $\beta$  cell death (Scheuner et al., 2005; Wang et al., 2005; Wang et al., 1999a). GLP-1R agonists such as exendin-4 maintain insulin biosynthesis and secretion while expanding islet mass



**Figure 4.** Exendin-4 accelerates recovery from ER stress-induced translational repression in INS-1 by potentiating the induction of the GADD34-PP1c eIF2 $\alpha$  phosphatase complex

Cultures were treated with vehicle alone (Veh or V) or 0.5  $\mu$ M thapsigargin (Tg or T) in the absence or presence of 50 nM exendin-4 (Ex-4 or E) or 20  $\mu$ M forskolin (Fk or F) for the indicated periods of time (**A** and **C**) or were exposed to vehicle alone or H89 for 20 min prior to and during a 4.5 hr (**B**) or 4 hr (**D**) treatment with thapsigargin in the

through direct effects on activation of  $\beta$  cell proliferation and inhibition of  $\beta$  cell death (Drucker, 2003; Kim et al., 2003; Li et al., 2003; Wang et al., 2004; Xu et al., 1999). GLP-1R agonists also lower blood glucose via multiple actions independent of the islet  $\beta$  cell, including reduction in gastric emptying, glucagon secretion and body weight. Hence, experiments demonstrating GLP-1R-dependent improvement in  $\beta$  cell function in rodents may reflect both indirect and direct aspects of GLP-1R action that converge on reduction of blood glucose and lipotoxicity. Although reduction of ER stress in peripheral insulin-sensitive tissues may also be associated with the amelioration of experimental diabetes (Ozcan et al., 2004), we now demonstrate that GLP-1R signaling pathways are directly coupled to modulation of the UPR and improved survival of ER-stressed  $\beta$  cells.

Currently available insulin secretagogues such as the sulfonylureas or the glitinides promote insulin exocytosis without acting more proximally to enhance insulin biosynthesis (Garcia et al., 1976; Schatz et al., 1975). In contrast, GLP-1R activation leads to induction of insulin gene transcription (Drucker et al., 1987) and increased pancreatic insulin biosynthesis (Hosokawa et al., 1996), leading to replenishment of  $\beta$  cell insulin content. GLP-1 is thought to increase insulin biosynthesis through induction of Pdx-1 transcription, leading to enhanced insulin gene expression (Wang et al., 1999b, 2001). Our data extend the concept of GLP-1 action in the  $\beta$  cell to control of translational repression downstream of PERK by attenuation of levels of phospho-eIF2 $\alpha$ , thereby promoting a faster resumption of protein synthesis during ER stress.

Our results are consistent with a sequence of events (Figure S7) whereby activation of the GLP-1R magnifies the ER stress-mediated induction of the transcriptional activator ATF-4 in a cAMP- and PKA-dependent manner. While we cannot exclude a role for signaling molecules such as EPAC as downstream mediators of the GLP-1R response (Holz, 2004), the observation that H89 abolished a number of actions of exendin-4 in the setting of ER stress implicates a major role for PKA in coupling GLP-1R actions to modulation of the UPR in INS-1 cells. We provide evidence strongly suggesting that stimulation of ATF-4 translation is the target for the early modulation of ER stress-mediated ATF-4 induction by GLP-1R activation in INS-1 cells, yet additional experiments will be required to further define whether this regulation occurs at the level of translation initiation and/or ribosomal transit time. The overinduction of ATF-4 early during the ER stress response would promote the ensuing enhanced upregulation of the downstream transcriptional targets CHOP and GADD34. ATF-4- and CHOP-mediated overinduction of GADD34, by significantly increasing the levels of the eIF2 $\alpha$  phosphatase complex, would initiate the subsequent later phase of exendin-4-dependent attenuation of phospho-eIF2 $\alpha$  levels, resulting in enhanced translational recovery. The higher protein synthesis rate during this later phase would further contribute to the sustained upregulation of ATF-4, GADD34, and CHOP but would also impact indirectly on the

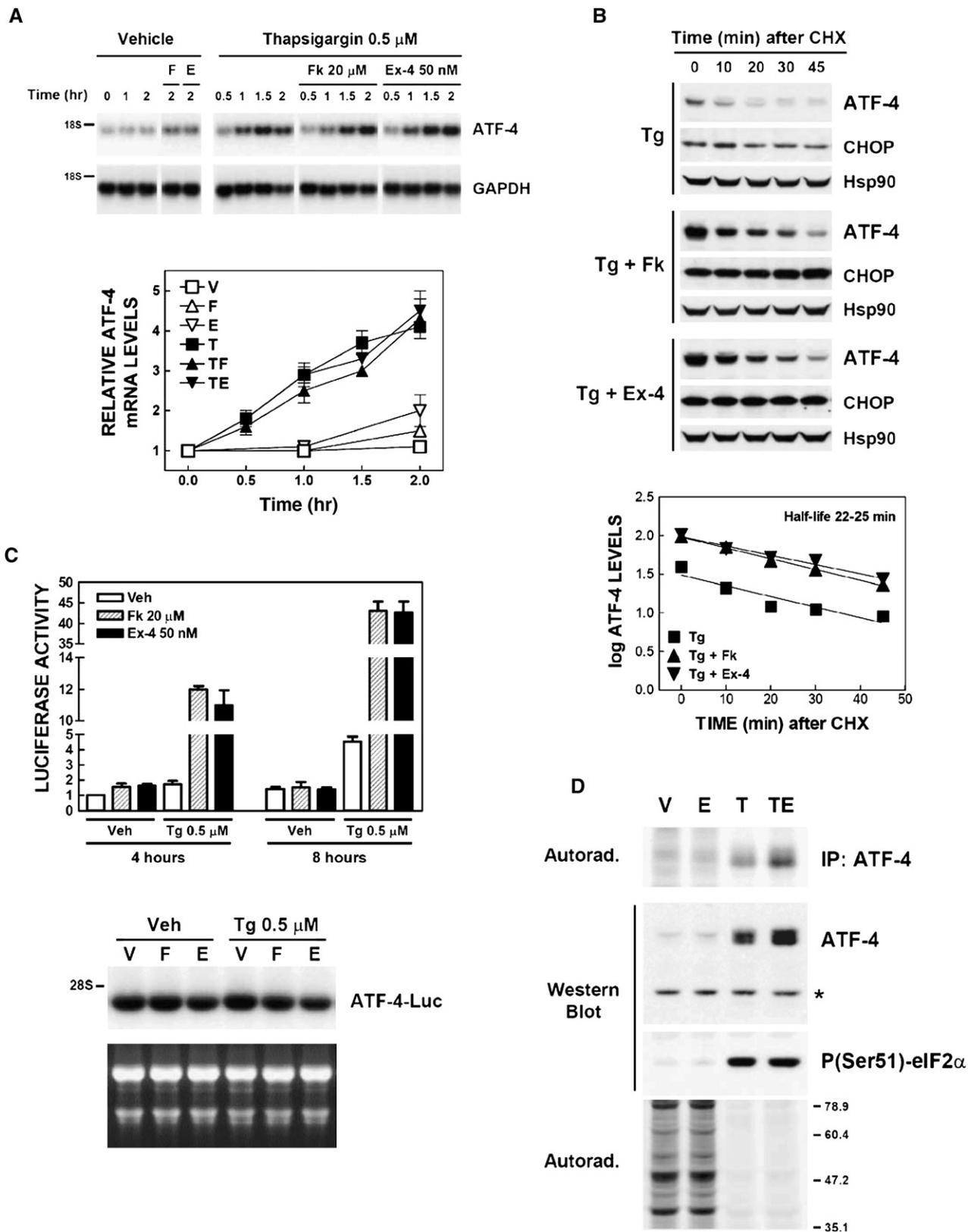
IRE1/XBP-1 arm of the UPR by facilitating the induction of the transcriptional activator sXBP-1.

We have shown that modulation of the activity of the PERK arm of the UPR by exendin-4 in  $\beta$  cells undergoing ER stress facilitates the shift from translational repression to the translational recovery phase. This shift is known to be required to implement the ER stress-induced gene expression program that transiently allows maintenance of ER homeostasis, thus promoting cell survival (Harding et al., 2000b). The same UPR can also contribute to cell death if activated persistently by sustained ER stress. Our findings explain the paradox of how GLP-1R signaling enhances both insulin biosynthesis and secretion while simultaneously reducing cell death in response to ER stress.

GLP-1R agonists have been shown to attenuate  $\beta$  cell death using rodent models of  $\beta$  cell apoptosis *in vivo* (Farilla et al., 2002; Li et al., 2003; Wang and Brubaker, 2002) and following a diverse number of experimental insults including exposure to cytotoxic agents, free fatty acids, and increased glucose *in vitro* (Buteau et al., 2004; Farilla et al., 2003; Wang et al., 2004). Collectively, activation of GLP-1R signaling increases levels of cAMP, Akt, and IRS-2 (Jhala et al., 2003), leading to reduced expression and activation of effector caspases. The data shown here extend the spectrum of antiapoptotic GLP-1 actions to ER stress-induced cell death, linking GLP-1R activation to cell survival via a cAMP and PKA-dependent pathway. Furthermore, it is possible that activation of related GPCRs also coupled to induction of cAMP in  $\beta$  cells may produce similar effects on the UPR. For example, we also demonstrated that GIP receptor activation mimics the actions of exendin-4 on INS-1 cells treated with thapsigargin, leading to reduction of eIF2 $\alpha$  phosphorylation and overinduction of ATF-4 and CHOP expression.

The diabetic  $\beta$  cell is required to chronically sustain a high level of proinsulin production while maintaining the molecular machinery required for accurate processing and subsequent release of mature insulin. Genetic mutations that impair ER function in the  $\beta$  cell lead to either accumulation of proinsulin in the ER (Yoshioka et al., 1997), defective upregulation of insulin biosynthesis in the face of rising glucose (Scheuner et al., 2005), or failure of translational attenuation (Harding et al., 2000b, 2001) and ultimately progressive  $\beta$  cell dysfunction, hyperglycemia, and apoptosis. Intriguingly, GLP-1R agonists increase first-phase insulin release and reduce the proinsulin:insulin ratio in diabetic subjects (Buse et al., 2004; Degn et al., 2004), indirect evidence for acute GLP-1R-dependent restoration of a more physiological secretory and processing pathway in islet  $\beta$  cells. Furthermore, our findings using isolated rat  $\beta$  cells or INS-1 cells demonstrate that exendin-4 directly relieves translational repression and increases insulin biosynthesis in the setting of ER stress via potentiation of the eIF2 $\alpha$  phosphatase complex. These findings may explain in part the GLP-1R-dependent restoration of  $\beta$  cell function observed in subjects with type 2 diabetes (Degn et al., 2004; Rachman et al., 1996; Zander et al., 2002). Remarkably, there is little current evidence linking GPCR

absence or presence of exendin-4 or forskolin. (A and B) Thirty minutes before harvesting, cells were labeled with [<sup>3</sup>H]leucine, and the incorporation of the tracer into 10% trichloroacetic acid precipitable protein was assessed by scintillation counting. Data are means  $\pm$  SD of triplicate cultures and are representative of four (A) and two (B) similar experiments. SD for data with coefficients of variation  $\leq$  4% is not visible on the graphs. (C and D) Whole-cell extracts were analyzed by immunoblotting for ATF-4, P(Ser51)-eIF2 $\alpha$ , and PP1c and in parallel subjected to microcystin affinity chromatography to purify PP1c-interacting proteins. Microcystin-agarose pull downs were immunoblotted with the indicated antibodies. The asterisk in (C) marks the position of an unidentified protein crossreacting with the anti-ATF-4 antibody. Results are representative of two independent experiments.



**Figure 5.** Exendin-4 stimulates ER stress-induced ATF-4 translation in INS-1 cells  
**(A)** Cells were treated with vehicle alone (V) or thapsigargin (T) in the absence or presence of exendin-4 (Ex-4 or E) or forskolin (Fk or F) for the indicated periods of time. Total RNA was then isolated and subjected to northern blot analysis. Blots were hybridized sequentially with radiolabeled ATF-4 and GAPDH (loading control) cDNA probes. The radioactive signal associated with ATF-4 was quantified by autoradiography on a storage phosphor screen, corrected by the signal from the loading control, and expressed relative to the values at time zero. Data are means  $\pm$  SEM from three to four independent experiments. SEM for data with coefficients of variation  $\leq$  4% is not visible on the graph.

activation to alleviation of ER stress. Our data suggest that coupling of GLP-1R signaling pathways to modulation of the ER stress response represents a feasible therapeutic strategy for simultaneous enhancement of  $\beta$  cell function and insulin biosynthesis while reducing the risk of cytotoxicity traditionally associated with sustained upregulation of ER function as a result of chronic demand for protein biosynthesis.

## Experimental procedures

### Materials

RPMI 1640 and DMEM tissue culture media were from HyClone, and fetal bovine serum (FBS) and antibiotics were from Invitrogen-GIBCO. Thapsigargin, tunicamycin, forskolin, phorbol 12-myristate 13-acetate (PMA), long R<sup>3</sup>IGF-1, cycloheximide, actinomycin D, 5,6-dichloro-1- $\beta$ -ribofuranosyl benzimidazole (DRB), protease inhibitor mixture (P-2714), and phosphatase inhibitor mixture I were purchased from Sigma. GLP-1(7–36)amide, exendin-4, and [D-Ala<sup>2</sup>]GIP were from California Peptide Research Inc. H89, PD98059, U0126, wortmannin, and LY294002 were obtained from Calbiochem. The expression vector pCMV-FlagVASP and the reporter plasmid TK-ATF4UTR-Luc were gifts from M.D. Uhler (University of Michigan, Ann Arbor, Michigan) and D. Ron (Skirball Institute of Biomolecular Medicine, New York, New York), respectively.

### Animal studies

Four-week-old female db/db (BKS.Cg-m<sup>+/+</sup>Lepr<sup>db</sup>/J) mice were purchased from Jackson Laboratories and were maintained on a 12 hr light/dark cycle with free access to standard rodent chow and water. All animal experiments were carried out in accordance with protocols and guidelines approved by the Toronto General Hospital Animal Care Committee. Commencing at 4 weeks of age, when all mice were normoglycemic, they were given twice-daily intraperitoneal (i.p.) injections of exendin-4 (24 nmol/kg) or phosphate-buffered saline (PBS) for 3 weeks and then sacrificed. Immediately prior to sacrifice, blood glucose levels were measured from a tail vein sample using a Glucometer Elite (Bayer). Pancreas samples were obtained for RNA extraction and immunohistochemistry. For pancreatic CHOP and insulin immunohistochemistry, the tissue was fixed in 4% paraformaldehyde and embedded in paraffin. Serial sections were stained with anti-CHOP/GADD153 (sc-575, Santa Cruz Biotechnology) or with anti-insulin (Dako Diagnostics) antibodies using standard protocols. The insulin immunopositive area was measured using a Leica DMR microscope equipped with a Leica DC 300F camera and the Leica QWin V3 software (Leica Microsystems Canada Inc.), and the number of cells exhibiting both nuclear CHOP and cytoplasmic insulin immunopositivity was determined.

### Culture of purified rat $\beta$ cells and rodent insulinoma cells, drug treatments, transient transfections, and luciferase assay

Purified  $\beta$  cells from adult Wistar rats were prepared as previously described (Pipeleers et al., 1985a). Freshly isolated  $\beta$  cells were reaggregated for 2 hr in a rotary shaking incubator (Pipeleers et al., 1985b) or seeded on polylysine-

coated microtiter plates (Pipeleers and Van de Winkel, 1986) for studies involving metabolic labeling or apoptosis assessment, respectively. Prior to use in experiments, purified  $\beta$  cells were precultured for 16 hr in Ham's F10 medium (Invitrogen-GIBCO) supplemented with 2 mM glutamine, 6.1 mM glucose, 0.5% (w/v) charcoal extracted bovine serum albumin, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Rat INS-1(832/13) cells (Hohmeier et al., 2000) were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 10 mM HEPES, 4 mM glutamine, 1 mM sodium pyruvate, and 50  $\mu$ M  $\beta$ -mercaptoethanol. The mouse insulinoma MIN6 cell line (Miyazaki et al., 1990) was grown in DMEM supplemented with 15% heat-inactivated FBS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 4 mM glutamine, and 50  $\mu$ M  $\beta$ -mercaptoethanol. Except where indicated, drug treatments were performed in regular growth medium. Exendin-4, GLP-1, [D-Ala<sup>2</sup>]GIP, and IGF-1 were dissolved in PBS and thapsigargin, tunicamycin, forskolin, PMA, kinase inhibitors, actinomycin D, DRB and cycloheximide in dimethyl sulfoxide. Final concentrations of drug solvent (vehicle) were identical in every culture irrespective of the particular treatment group. INS-1 cells were transfected using FuGene (Roche) or Lipofectamine 2000 (Invitrogen-GIBCO) and utilized for experiments 24 hr later. Luciferase assays were performed as previously described (Koehler et al., 2005).

### Western blot analysis

Following drug treatment as indicated, cells were washed in ice-cold PBS and lysed in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) supplemented with protease and phosphatase inhibitor mixtures (both at 1:100 dilution), 10  $\mu$ g/ml pepstatin A, 5 mM sodium fluoride, and 500  $\mu$ M sodium orthovanadate. Cell lysates were cleared by centrifugation and boiled for 5 min in sample buffer containing  $\beta$ -mercaptoethanol. Protein concentration was determined using the BCA protein assay (Pierce). Forty to fifty micrograms of cell lysate protein was resolved by SDS-PAGE and immunoblotted as described (Koehler et al., 2005). The following primary antibodies were used: rabbit polyclonal antibodies to ATF-4 (sc-200), XBP-1 (sc-7160), GADD34 (sc-825) and CHOP (sc-575) (Santa Cruz Biotechnology) at 1:500 dilutions; rabbit polyclonal antibodies reactive to phospho-eIF2 $\alpha$  Ser51, phospho-PERK Thr980, Akt, phospho-Akt Ser473, phospho-VASP Ser157, Erk1/2 mitogen-activated protein kinase, and phospho-Erk1/2 Thr202/Tyr204 (Cell Signaling Technologies) at 1:1000 dilutions; and anti-PP1c antibody (Upstate Cell Signaling Solutions) at 1:2000 dilution and mouse monoclonal anti-Hsp90 (BD Biosciences, Mississauga, Ontario) at 1:3000 dilution. Densitometry was performed on blots exposed to Biomax MR film (Eastman Kodak Co.) using a Hewlett-Packard ScanJet 5300C scanner and NIH Image software.

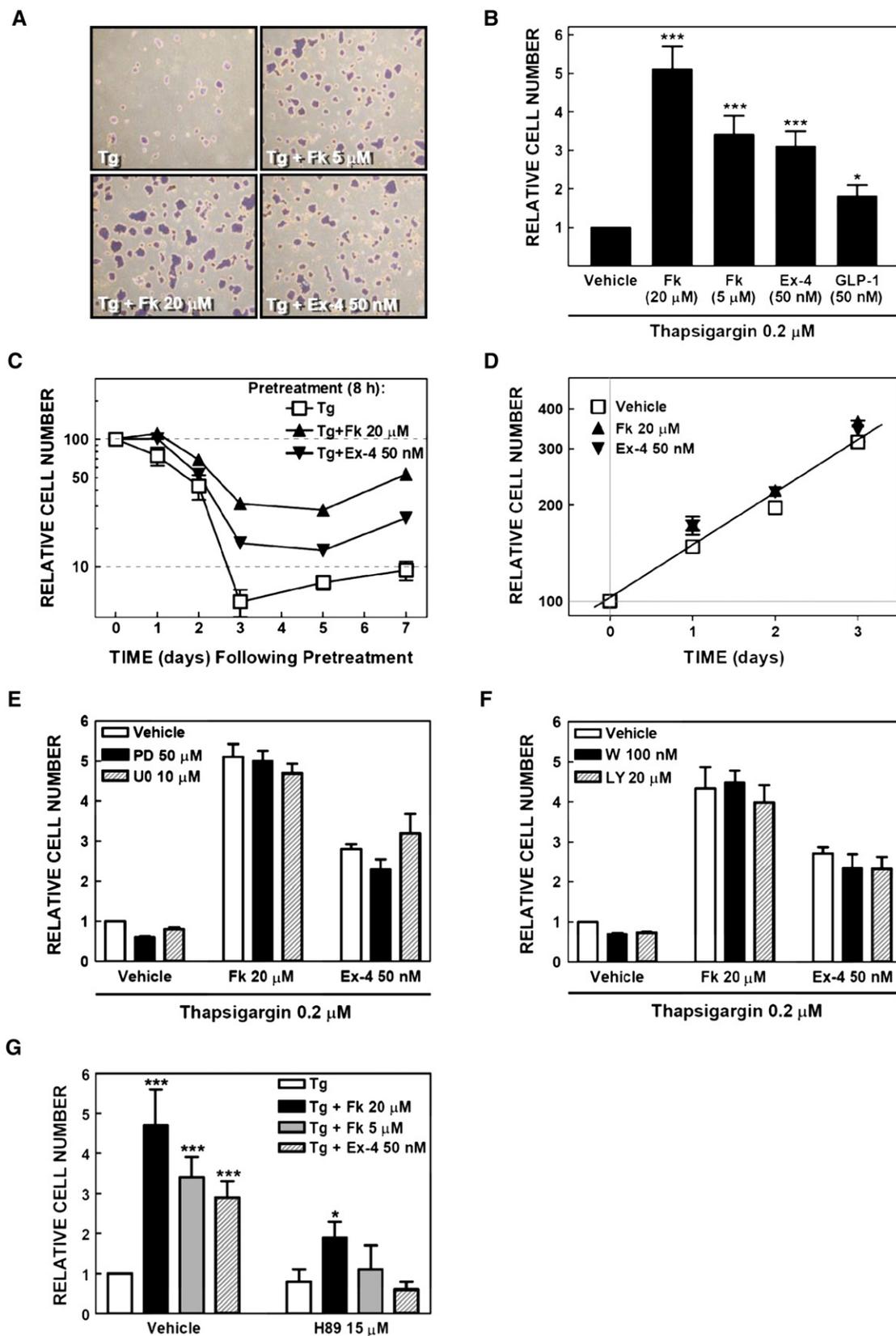
### Microcystin affinity chromatography

Cells were lysed in a buffer containing 137 mM NaCl, 20 mM Tris (pH 7.6), 0.25% Nonidet P-40, 5 mM NaF, 500  $\mu$ M sodium orthovanadate, and protease inhibitor mixture (1:100 dilution). Lysates were cleared by centrifugation and their protein concentration determined. Four hundred micrograms of cell lysate protein was incubated at 4°C for 4 hr with microcystin-agarose (Upstate Cell Signaling Solutions). Beads were then washed four times with

(B) Cells were exposed to 0.5  $\mu$ M thapsigargin (Tg) in the absence or presence of 50 nM exendin-4 or 20  $\mu$ M forskolin for 4 hr. Cycloheximide (CHX) 80  $\mu$ M was then added to the cultures and the incubation continued for the indicated periods of time. Total cell extracts were analyzed by immunoblotting for ATF-4, CHOP, and Hsp90. The intensity of the ATF-4 signal was quantified by densitometry, corrected by the loading control, and expressed relative to the values at time zero. Data are average of two independent experiments. ATF-4 half-life was calculated from the logarithmically transformed best-fit line by linear regression analysis. Similar results were obtained when the rate of ATF-4 decay was examined following a 1 hr treatment with thapsigargin with or without exendin-4 or forskolin (data not shown).

(C) Twenty-four hr after transfection with the reporter plasmid TK-ATF4UTR-Luc, cells were exposed to vehicle alone (Veh) or thapsigargin (Tg) in the absence or presence of exendin-4 or forskolin for 4 and 8 hr. Duplicate cultures from each treatment group were used to determine luciferase activity (top) and in parallel ATF-4-luciferase mRNA (ATF-4-Luc) expression (bottom). Luciferase activity is expressed relative to the activity of cells treated with vehicle alone for 4 hr following normalization for protein content. Data are means  $\pm$  SD of quadruplicate cultures and are representative of five similar experiments. Levels of ATF-4-Luc transcripts were assessed by northern blot analysis of total RNA isolated from cells treated as indicated for 8 hr. Blots were hybridized with a radiolabeled luciferase cDNA probe. Ethidium bromide staining of the northern blot is shown as a control for loading and integrity of RNA. Results are representative of two independent experiments.

(D) Cultures were treated with vehicle alone or 0.5  $\mu$ M thapsigargin in the absence or presence of 50 nM exendin-4 for 45 min prior to and during a 15 min metabolic pulse labeling with [<sup>35</sup>S]methionine/cysteine. Whole-cell lysates were subjected to immunoprecipitation (IP) with an ATF-4-specific antibody, and the immune complexes were resolved by SDS-PAGE, transferred onto nitrocellulose, and revealed by autoradiography on a storage phosphor screen (top panel). An equal amount of the total cell lysates from the same groups of radiolabeled cells was also fractionated by SDS-PAGE followed by immunoblotting with anti-ATF-4 and anti-P(Ser51)-eIF2 $\alpha$  antibodies (middle panels) and lastly autoradiography (bottom panel) to quantify global protein synthesis rates. An unidentified protein (asterisk) crossreacting with the anti-ATF-4 antibody is shown to document equal loading. Molecular mass standards (in kDa) appear on the right. Results are representative of four independent experiments.



**Figure 6.** GLP-1R signaling enhances INS-1 survival following an ER stress challenge

Cells were pretreated with 0.2  $\mu$ M thapsigargin (Tg) in the absence or presence of exendin-4 (Ex-4), GLP-1, or forskolin (Fk) for 8 hr. Drugs were then removed and the cells allowed to resume growth in normal media for 5 days (**A and B**) or for up to 7 days (**C**). Photomicrographs illustrated in (**A**) were taken under phase contrast following crystal violet staining of the cultures. Magnification, 50 $\times$ . Cell number was assessed at the indicated time points by using a tetrazolium salt bioreduction assay and expressed

ice-cold lysis buffer and boiled in reducing sample buffer prior to SDS-PAGE and blotting as described above.

#### Metabolic labeling of newly synthesized proteins and immunoprecipitation

To measure insulin synthesis in purified rat  $\beta$  cells, cultures were labeled for 1 hr in Ham's F10 medium with 0.5% BSA and 250  $\mu$ Ci/ml of L-[3,5- $^3$ H]tyrosine (40–60 Ci/mmol, Amersham) followed by immunoprecipitation of  $^3$ H-labeled proteins with anti-insulin serum as previously described (Schuit et al., 1991). To evaluate general translation rates in INS-1 cells, cultures were labeled in normal growth medium for 30 min with 4–5  $\mu$ Ci/ml of L-[4,5- $^3$ H]leucine (153 Ci/mmol) (Amersham Biosciences). The cells were harvested in 1 M NaOH, and the protein was precipitated with trichloroacetic acid (10% final concentration) and recovered by centrifugation. The radioactivity associated with the pellet was measured by scintillation counting. Protein content of the pellet was determined using the Bio-Rad Protein Assay (Bio-Rad). To assess ATF-4 synthesis in INS-1 cells, the regular culture medium was replaced by medium containing 10% of the normal methionine and cysteine content 15 min before the addition of [ $^{35}$ S]Met/Cys EasyTag Express protein labeling mix (PerkinElmer Life Sciences) at 200  $\mu$ Ci/ml for 15 min. Cells were lysed in 0.5 M NaCl-containing RIPA buffer and the lysate cleared by centrifugation, diluted to 0.3 M NaCl, and subjected to immunoprecipitation with a polyclonal rabbit anti-ATF-4 antiserum (either sc-200 from Santa Cruz Biotechnology or obtained from D. Ron) prebound to protein A agarose (Pierce). Radiolabeled immune complexes were resolved by SDS-PAGE, electrotransferred to nitrocellulose, and revealed by autoradiography on a storage phosphor screen. Radioactive signal quantification was performed using a Storm imaging system and the ImageQuant software (GE Healthcare).

#### RNA isolation, northern blot analysis, and quantitative real-time RT-PCR

Total RNA from pancreas samples or from INS-1 cells was extracted by the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). For northern blotting, 5  $\mu$ g of RNA was electrophoresed in a 1% agarose-formaldehyde gel, transferred to a Nytran membrane (Schleicher & Schuell), and UV crosslinked. Blot hybridization with [ $^{32}$ P]-radiolabeled cDNA probes was performed in ExpressHyb buffer (BD Biosciences) as recommended by the manufacturer. Template DNA for the probes was generated by RT-PCR (GAPDH; see Yusta et al. [2000]) for primer sequences) or was excised from IMAGE consortium cDNA clones purchased from the American Type Culture Collection (ATF-4, IMAGE:4992862; c-FOS, IMAGE:3688670). Luciferase template DNA was excised from the TK-ATF4UTR-Luc reporter plasmid. After extensive washing, blots were subjected to autoradiography on a storage phosphor screen. Quantification of the transcript levels was performed as indicated above. First-strand cDNA synthesis was performed with 5  $\mu$ g of DNase I-treated total RNA using random hexamers and SuperScript II (Invitrogen-GIBCO) according to the manufacturer's instructions. Duplicate aliquots of the first-strand reactions were used as templates for real-time quantitative PCR using TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix (Applied Biosystems) on an ABI PRISM 7900HT Sequence Detection System. Relative quantification of transcript levels was performed by the  $2^{-\Delta C_t}$  method (Livak and Schmittgen, 2001) using the  $C_t$  values obtained from the PCR amplification kinetics with the ABI PRISM SDS 2.1 software.

#### Measurement of XBP-1 mRNA splicing

cDNA generated from total RNA as described above was used as template for PCR with primers flanking the intron excised by IRE1 from the XBP-1

mRNA. Primer sequences used to amplify rat XBP-1 were 5'-AACAGAGTAGCAGCACAGACTGC-3' and 5'-TCCTTCTGGGTAGACCTCTGGGAG-3'. The sequence of the primers utilized to amplify mouse XBP-1 has been previously reported (Iwawaki et al., 2004). Amplification was performed at an annealing temperature of 60–63°C for 30 cycles. PCR products were separated by electrophoresis on a 3% agarose gel and visualized by ethidium bromide staining. Unspliced rat XBP-1 gave a product of 480 bp, and the spliced rat cDNA was 454 bp. Unspliced mouse XBP-1 gave a product of 205 bp, and the spliced mouse cDNA was 179 bp. The percentage of sXBP-1 to total XBP-1 mRNA was determined by densitometry using NIH Image software on images of the ethidium bromide-stained gels acquired with a gel documentation system.

#### Quantification of apoptosis in purified rat $\beta$ cell cultures

Living and dead  $\beta$  cells were visualized by fluorescence microscopy following propidium iodide and HOECHST 3342 staining (Hoorens et al., 1996). Viable cells were identified by their intact nuclei with blue fluorescence, necrotic cells by their intact nuclei with yellow-red fluorescence, and apoptotic cells by their fragmented nuclei with either blue (early apoptosis) or yellow-red (late apoptosis) fluorescence (Hoorens et al., 1996). The apoptotic index is defined by the following equation: (% apoptosis in drug-treated cultures – % apoptosis in vehicle-treated cultures)/(100 – % apoptosis in vehicle-treated cultures)  $\times$  100.

#### Cell survival assays and colony outgrowth assessment in INS-1 cells

Cells were exposed to thapsigargin in the presence or absence of the specified agonists and/or inhibitors for 8 hr. At the indicated periods of time following treatment, the number of viable cells in each condition was evaluated by measuring the bioreduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt at 490 nm using the Cell-Titer 96 aqueous assay (Promega). Crystal violet staining was used to reveal cell colony outgrowth 5 days after the thapsigargin challenge. Phase contrast images of representative areas of the stained cultures were acquired with a Leica DC 300F camera mounted on a Leica DMIRB inverted microscope and the Leica IM500 software.

#### Statistical analysis

Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison posthoc test by using GraphPad Prism 3.03 (GraphPad Software Inc.).

#### Supplemental data

Supplemental data include seven figures and Supplemental references and can be found with this article online at <http://www.cellmetabolism.org/cgi/content/full/4/5/391/DC1>.

#### Acknowledgments

The work in this manuscript is supported in part by operating grants from the Juvenile Diabetes Research Foundation, the Canadian Diabetes Association, and the National Institutes of Health. D.J.D. is supported by a Canada Research Chair in Regulatory Peptides, and J.L.E. is supported by a CIHR Canadian Graduate Scholarship Doctoral Award and a grant from the Banting and Best Diabetes Centre/Novo-Nordisk. J.A.K. is supported by a postdoctoral fellowship award from the Banting and Best Diabetes Centre and the McLaughlin Center for Molecular Medicine (MCM). We thank Cameron Scott and Sergio Grinstein for assistance with calcium imaging experiments.

relative to the cell number in the thapsigargin alone-treated cultures (**B**) or as a percentage of the cell number prior to the thapsigargin treatment (**C**). Data in (**B**) are means  $\pm$  SE ( $n = 3$  to 8 independent experiments, each performed in quadruplicate). Statistical comparisons are relative to the thapsigargin alone-treated cultures (\* $p < 0.05$ , \*\*\* $p < 0.001$ ). Data plotted in (**C**) are means  $\pm$  SD of quadruplicate determinations from one representative experiment of three with similar results. SD for data with coefficients of variation  $\leq 4\%$  is not visible on the graph. (**D**) Following an 8 hr exposure of the cells to vehicle alone, exendin-4, or forskolin, media were replaced and cell number determined at the indicated time points as described above. 100 refers to the cell number at the beginning of the treatments. Data (means  $\pm$  SD of quadruplicate determinations) are representative of three similar experiments. (**E–G**) Cultures were exposed to vehicle alone, PD98059 (PD), or U0126 (U0) (**E**), wortmannin (W) or LY294002 (LY) (**F**), or H89 (**G**) for 20 min prior to and during an 8 hr treatment with 0.2  $\mu$ M thapsigargin in the absence or presence of exendin-4 or forskolin. Seven days after the thapsigargin challenge, cell number was determined and expressed relative to the cell number in the cultures treated with thapsigargin alone. Data shown in (**E**) and (**F**) are means  $\pm$  SD of representative experiments performed in quadruplicate and repeated twice. Data shown in (**G**) are means  $\pm$  SE ( $n = 4$  independent experiments each performed in quadruplicate). Statistical comparisons are relative to the corresponding cultures treated by thapsigargin alone (\* $p < 0.05$ , \*\*\* $p < 0.001$ ).

D.J.D. is a member of the Scientific Advisory Board of Amylin Pharmaceuticals, Inc., and Eli Lilly Inc.

Received: January 4, 2006  
 Revised: August 16, 2006  
 Accepted: October 6, 2006  
 Published: November 7, 2006

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