Exendin-4 stimulates islet cell replication via the IGF1 receptor activation of mTORC1/S6K1

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Abstract

Glucagon-like peptide 1 receptor (GLP1R) agonists, such as exendin-4, potentiate glucose-stimulated insulin secretion and are currently used in the management of type 2 diabetes. Interestingly, GLP1R agonists also have the ability to augment β -cell mass. In this report, we provide evidence that in the presence of glucose, exendin-4 stimulates rodent islet cell DNA replication via the activation of ribosomal protein S6 kinase 1 (S6K1) and that this is mediated by the protein kinase B (PKB)-dependent activation of mTOR complex 1 (mTORC1). We show that activation of this pathway is caused by the autocrine or paracrine activation of the IGF1 receptor (IGF1R), as siRNA-mediated knockdown of the IGF1R effectively blocked exendin-4-stimulated PKB and mTORC1 activation. In contrast, pharmacological inactivation of the epidermal growth factor receptor has no discernible effect on exendin-4-stimulated PKB or mTORC1 activation. Therefore, we conclude that GLP1R agonists stimulate β -cell proliferation via the PKB-dependent stimulation of mTORC1/S6K1 whose activation is mediated through the autocrine/paracrine activation of the IGF1R. This work provides a better understanding of the molecular basis of GLP1 agonist-induced β -cell proliferation which could potentially be exploited in the identification of novel drug targets that increase β -cell mass.

Key Words

- ▶ mTORC1
- ▶ PKB/AKT
- ▶ β -cell
- ▶ IGF1R
- ▶ GLP1

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Introduction

Type 2 diabetes mellitus (T2DM) is a disorder that is characterized by high blood glucose in the context of insulin resistance and relative insulin deficiency (Lingohr *et al.* 2002, Prentki & Nolan 2006). It causes metabolic changes that lead to the damage and functional impairment of organs and tissues, resulting in increased morbidity and mortality. It is this form of diabetes whose prevalence is increasing at an alarming rate due to the 'obesity epidemic'. This is because adiposity is associated with insulin resistance, an important

contributing factor in the development of T2DM. However, although the majority of people who are obese are insulin resistant, they maintain normoglycemia through an increase in insulin secretion, which is sustained through increased β -cell function and mass. This increase in β -cell mass is thought to occur through β -cell hypertrophy, neogenesis, proliferation, and a decrease in the rate of apoptosis (Lingohr *et al.* 2002, Prentki & Nolan 2006). T2DM develops when β -cells fail to adequately compensate for the increase in insulin

demand. Importantly, exendin-4 (Parker *et al.* 1984, Eng *et al.* 1992), a glucagon-like peptide 1 receptor (GLP1R) agonist developed for the management of T2DM, potentiates glucose-dependent insulin secretion and stimulates increased β -cell mass through promotion of β -cell survival, differentiation, and proliferation (Xu *et al.* 1999, Wang & Brubaker 2002, Wrede *et al.* 2002, Li *et al.* 2005, Cornu *et al.* 2009).

Previous studies have implicated both cAMP- (Buteau *et al.* 2003, Song *et al.* 2008) and β -arrestin (Talbot *et al.* 2012)-dependent mechanisms in the proliferative effects of GLP1 on pancreatic β -cells. In addition, it has been reported that the proliferative action of GLP1 is mediated by the transactivation of epidermal growth factor receptor (EGFR; Buteau *et al.* 2003) or the increased expression and activation of the insulin-like growth factor 1 receptor (IGF1R) (Cornu *et al.* 2010) both of which result in the activation of the PI3K/protein kinase B (PKB) pathway. Many of the signalling pathways activated upon GLP1R activation have the potential to signal through mTOR complex 1 (mTORC1); a serine threonine kinase known to be activated by GLP1 (Briaud *et al.* 2003, Kwon *et al.* 2004, Moore *et al.* 2009) and whose hyperactivation stimulates β -cell mass (reviewed in Xie & Herbert (2012)). The best-characterized targets of mTORC1 are the eIF4E-binding proteins (4EBPs) and the ribosomal protein S6 kinases 1/2 (S6K1/2) (Proud 2007). Phosphorylation of 4EBP by mTORC1 results in the release of 4EBP from eIF4E and an increase in the translation of highly cap-dependent mRNAs important in cell cycle progression, such as cyclin-D1 (Averous & Proud 2006, Averous *et al.* 2008). S6K1/2 phosphorylates a large number of substrates implicated in the control of metabolism and cell growth (Xie & Herbert 2012). In β -cells, S6K1 appears to play an important role in the regulation of β -cell growth, as β -cell-specific S6K1 knockout mice have comparatively smaller β -cells compared with WT mice, which coincides with hypoinsulinemia and impaired glucose tolerance (Pende *et al.* 2000, Ruvinsky *et al.* 2005).

Our aim was to investigate how exendin-4 stimulates β -cell proliferation. To these ends, we have demonstrated, in rat islets of Langerhans, that in the presence of glucose, exendin-4 acutely activates mTORC1 via the autocrine/paracrine activation of the IGF1R, whereas EGFR, which was previously shown to stimulate β -cell proliferation, is not involved in this process. Importantly, we have provided evidence that the positive effect of exendin-4 on β -cell proliferation is mediated through the mTORC1 downstream target S6K1 rather than 4EBP1 or the stimulation of protein synthesis, which unravels a

previously unreported role of S6K1 on the replication of pancreatic β -cells.

Materials and methods

Materials

Foetal bovine serum (FBS) was purchased from Invitrogen. [35 S]-methionine/cysteine was purchased from Amersham Pharmacia Biotech. All other chemicals and reagents were purchased from Sigma-Aldrich, unless otherwise stated.

Islet isolation and islet cell culture

Male Wistar albino rats weighing 200–250 g were used for the isolation of pancreatic islets. Islets were isolated and dispersed as described previously (Moore *et al.* 2011). Clusters and isolated cells were then cultured in 24-well plate pre-coated with poly-D-lysine at 37 °C in humidified 5% CO₂ in the RPMI-1640 medium supplemented with 10% (v/v) heat inactivated FBS and 100 μ g/ml streptomycin, 100 units/ml penicillin sulfate, and 100 units/ml neomycin.

Cell culture

Rat insulinoma-derived INS1E cells (Merglen *et al.* 2004) were used between passages 65 and 90 at ~80% confluence. The cells were maintained in a humidified incubator (95% air and 5% CO₂) at 37 °C in RPMI-1640 (Sigma), supplemented with 11 mM glucose, 2 mM L-glutamine, 5% (v/v) FBS, 1 mM sodium pyruvate, 10 mM HEPES, 50 μ M β -mercaptoethanol, 100 μ g/ml streptomycin, 100 units/ml penicillin sulfate, and 100 units/ml neomycin.

Proliferation assay

Proliferation assays were carried out essentially as described in the study by Kwon *et al.* (2006) with modification. Dispersed islet cells were incubated in RPMI minus glucose media supplemented with 0.2% FBS and either 2 or 7.8 mM glucose in the absence or presence of 10 nM exendin-4 for 96 h. The media were replaced every 24 h and the cells labeled for the last 24 h by the addition of 10 μ Ci/ml [3 H]-thymidine. Where indicated inhibitors were added for the last 24 h. The cells were then washed thrice with 1 $\times$ PBS before the addition of 5% trichloroacetic acid (TCA) for 30 min. The cell lysates

were then collected and centrifuged at 16 000 *g*, at 4 °C for 10 min. The pellets were then washed twice with 5% TCA before the addition of 0.1 M NaOH for 30 min at room temperature. Scintillant was then added to each sample and [³H]-thymidine incorporation determined by scintillation counting using the LS6400 multi-purpose scintillation counter.

siRNA transfection of INS1E cells and dispersed islets

Transfection with siRNA was carried out using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. INS1E cells or dispersed islets were seeded into 24-well plates 1 day before transfection in 500 µl of growth medium without antibiotics. For knockdown of insulin receptor (INSR) and IGF1R, the cells were transfected for 72 h with 100 nm of on-target plus siRNA (J-091936-10-0010, rat IGF1R, NM_052807 and J-080102-10-0010, rat INSR, NM_017071, Dharmacon). siGENOME non-targeting siRNA (Dharmacon, GE Healthcare Life Sciences, Piscataway, NJ, USA) was used as a negative control. The cells were then serum starved overnight in CMRL-1066 medium containing glutamine before treatments.

SDS-PAGE and western blotting analysis

All treatments were stopped by the addition of ice-cold Triton lysis buffer (1% Triton, 10 mM β-glycerophosphate, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM benzamidine HCl, 0.2 mM phenylmethylsulfonyl fluoride, 1 µg/ml each of leupeptin and pepstatin, 0.1% β-mercaptoethanol, and 50 mM sodium fluoride). The lysates were then centrifuged for 10 min at 16 000 *g*. The pellet was discarded and the supernatants were stored at −80 °C. Protein concentrations were determined by the Bradford assay (Bio-Rad). SDS-PAGE and western blotting analysis were carried out as described previously (Gomez *et al.* 2008). Phospho (P)-PKB (S473), P-rpS6 (S240/244), P-PRAS40 (T246), P-TSC2 (T1462), P-GSK3α (S9/21), P-Erk1/2 (T202/Y204), and P-S6K1 (T389) were purchased from Cell Signalling Technology (Beverly, MA, USA). Anti-INSR β-subunit, anti-IGF1R β-subunit, and anti-PKB and anti-rpS6 antibodies were purchased from Santa Cruz Biotechnology. Anti-rabbit IgG HRP-linked antibody was purchased from New England Biolabs (Hitchin, UK). Detection was by HRP-linked anti-rabbit secondary antibodies and ECL (GE Healthcare, Bucks, UK). Intensities of western blotting bands were quantified using the freely available Image J Software (version 1.47).

7-Methyl GTP pulldown

After treatments, islets were lysed in ice-cold Triton lysis buffer. Protein lysates were precleared by centrifugation for 10 min at 16 000 *g* before binding to 7-methyl GTP (m7GTP) sepharose 4B beads (Amersham Biosciences) for 1 h at 4 °C. The beads were then washed twice with Triton lysis buffer and resuspended in 2× Laemmli sample buffer before subjected to SDS-PAGE.

Protein synthesis measurements

The cells were incubated in the presence of 20 µCi [³⁵S]-methionine/cysteine per ml. They were then lysed in ice-cold Triton lysis buffer and 20 µg proteins were spotted onto the squares of 3 MM Whatman paper. The papers were washed twice in boiling 5% TCA-containing 0.1 g/l L-methionine for 1 min, washed once using 5% TCA-containing 0.1 g/l L-methionine, and once with absolute ethanol. The papers were dried, transferred to a scintillation tube containing emulsifier-safe, and count per minute was determined by scintillation counting using a LS6400 multi-purpose scintillation counter (Beckman Coulter, Brea, CA, USA).

Immunofluorescence and DNA staining

Dispersed primary rat islets were plated on coverslips. After treatments, the cells were fixed for 15 min with 4% paraformaldehyde diluted in 1× PBS and then washed three times for 5 min in 1× PBS. The coverslips were then incubated in blocking buffer (1× PBS+5% BSA+0.3% Triton X-100) for 60 min before incubation with anti-insulin antibody (Cell signalling Technology) diluted in antibody dilution buffer (1× PBS+1% BSA+0.3% Triton X-100) overnight at 4 °C. The coverslips were washed three times for 5 min in 1× PBS before being incubated with fluorochrome-conjugated secondary anti-rabbit antibody for 1 h at RT in the dark. For staining of nuclear DNA, coverslips were incubated in 1× PBS containing 2.5 µg/ml propidium iodide for 15 min at RT in the dark. Fluorescence/stained cells were imaged using laser confocal microscopy.

Statistical analysis

All data are expressed as means ± S.E.M. (*n* = 3) and analyzed by one-way ANOVA followed by Bonferroni's correction for all pair-wise comparisons. Significance was assigned as **P* = 0.05–0.01, ***P* = 0.01–0.001, and ****P* < 0.001.

Results

Exendin-4 stimulates islet cell replication via a rapamycin-sensitive mechanism

To investigate the role of mTORC1 in GLP1R agonist-stimulated β -cell replication, dispersed islets were cultured in either 2 or 7.8 mM glucose in the presence or absence of

exendin-4 (a GLP1R agonist), and the incorporation of [3 H]-thymidine into DNA in the presence and absence of rapamycin (a selective inhibitor of mTORC1) was determined (Fig. 1A). Compared with cells incubated in 2 mM glucose, those incubated in 7.8 mM glucose caused a small increase in DNA replication and this was significantly potentiated by exendin-4. The addition of

Figure 1

Exendin-4 (Ex.4) enhances islet cell proliferation via a rapamycin (Rap.)-sensitive mechanism. (A) Dispersed rat islets were incubated in RPMI in the presence of [3 H]-thymidine at either 2 mM glucose (Glu.), 7.8 mM Glu., or 7.8 mM Glu. plus 10 nM Ex.4 for 96 h. Where indicated, 200 nM Rap. were added for the last 24 h. [3 H]-thymidine incorporation into DNA was determined and the results were plotted as percentage of 2 mM Glu. control. Below are representative morphological images of the islet cultures after treatments and an immunofluorescence confocal image of dispersed islets cultured for 96 h with Glu. plus Ex.4 and probed with antibodies to insulin (green) and stained with propidium iodide (red). (B and C) Rat islets of Langerhans were incubated in RPMI containing

[35 S]-methionine/cysteine and supplemented with either 2 mM Glu., 7.8 mM Glu., or 7.8 mM Glu. plus 10 nM Ex.4, in the presence or absence of 200 nM Rap. for 2 h. The cells were lysed and (B) 4EBP1 cap-binding activity was determined by m7GTP affinity chromatography followed by SDS-PAGE and western blotting using antisera against 4EBP1, eIF4E, phosphorylated (P)-rpS6 (S240/244), P-PKB (S473), and, as a loading control, rpS6. Top panel: total lysate. Bottom panel: m7GTP pulldown. SE, short exposure and LE, long exposure. (C) [35 S]-methionine/cysteine incorporation into protein was determined by TCA precipitation followed by scintillation counting. * $P=0.05-0.01$, ** $P=0.01-0.001$, *** $P<0.001$.

rapamycin significantly inhibited exendin-4 plus glucose-stimulated DNA replication. Morphological images indicate that almost all of the cells cultured were islet cells (Fig. 1A). Using Immunofluorescence, we demonstrate that the vast majority of these cells are insulin-expressing β -cells. However, we cannot exclude the possibility that we are also measuring changes in the proliferation of other islet and non-islet cells. To investigate the effects of these treatments on the activation of mTORC1 and mTORC2, the phosphorylation status of rpS6 on S240/244 and 4EBP1 (read-outs of mTORC1 activity) and the phosphorylation of PKB on S473 (a read-out of mTORC2 activity) were determined by western blotting analysis (Fig. 1B). Compared with cells incubated in 2 mM glucose, those in 7.8 mM glucose stimulated the phosphorylation of PKB and of rpS6. Moreover, 7.8 mM glucose caused a small increase in the hyperphosphorylated (γ) form of 4EBP1. The addition of exendin-4 significantly potentiated the phosphorylation of rpS6 and PKB, but caused little change in the amount of the hyperphosphorylated form of 4EBP1. As anticipated, the addition of rapamycin inhibited exendin-4 plus glucose-stimulated rpS6 phosphorylation and caused an increase in the hypo-phosphorylated (α) form of 4EBP1. However, rapamycin treatment also led to a decrease in PKB phosphorylation on S473, presumably via the inactivation of mTORC2 (Barlow *et al.* 2012).

The hyper-phosphorylation of 4EBP1 by mTORC1 promotes the dissociation of 4EBP1 from eIF4E, which increases the rate of cap-dependent translation (Beretta *et al.* 1996) and which has been reported to promote cell proliferation. Therefore, we also investigated the association of eIF4E with 4EBP1 by m7GTP affinity chromatography (Fig. 1B). 7.8 mM glucose or 7.8 mM glucose plus exendin-4 had no detectable effect on the association of eIF4E with 4EBP1. However, rapamycin, as expected, significantly increased the association of 4EBP1 with eIF4E.

As mTORC1 also classically stimulates protein synthesis, an important hypertrophic stimulus, the effects of exendin-4 on the rate of protein synthesis were also examined: 7.8 mM glucose significantly stimulated the rate of total protein synthesis (Fig. 1C). However, we could not detect any significant potentiation of protein synthesis by exendin-4.

Taken together, these results provide evidence that exendin-4 potentiates glucose-stimulated islet cell proliferation via an mTOR-dependent pathway which does not parallel changes in the rate of total protein synthesis or the dissociation of 4EBP1 from eIF4E.

Evidence that exendin-4-stimulated islet cell proliferation requires the activation of S6K1

S6K1 is a key downstream target of mTORC1, which has been shown to be crucial in the maintenance of β -cell size (Pende *et al.* 2000, Ruvinsky *et al.* 2005). However, it is unknown whether the inhibition of S6K1 has any impact on β -cell replication. Therefore, we investigated the role of S6K1 in exendin-4-induced islet cell proliferation. Dispersed rat islets of Langerhans were cultured in 2 mM glucose, 7.8 mM glucose, or 7.8 mM glucose plus exendin-4 in the presence or absence of the highly selective S6K1 inhibitor PF4708671 (n.b. S6K1: IC₅₀ is 0.16 μ M/S6K2: IC₅₀ is 65 μ M; Bilanges & Vanhaesebroeck 2010, Pearce *et al.* 2010) and the incorporation of [³H]-thymidine into DNA was determined (Fig. 2A). Compared with islet cells incubated in 2 mM glucose, those in 7.8 mM glucose plus exendin-4 stimulated DNA replication. The addition

Figure 2

S6K1 is required for exendin-4-stimulated cell proliferation. (A) Dispersed rat islets of Langerhans were incubated in RPMI containing 2 mM glucose, 7.8 mM glucose, or 7.8 mM glucose plus 10 nM exendin-4 for 96 h. [³H]-thymidine and, where indicated, PF4708671 were added during the last 24 h. DNA was extracted by TCA precipitation and [³H]-thymidine incorporation determined using a scintillation counter. * $P=0.05-0.01$, ** $P=0.01-0.0011$. (B) Rat islet of Langerhans were incubated in RPMI containing 2 mM glucose, 7.8 mM glucose, or 7.8 mM glucose plus 10 nM exendin-4 in the presence or absence of PF4708671. The islets were then lysed and the proteins separated by SDS-PAGE and western blotted using antisera against phospho (P)-PKB (S473) and P-rpS6 (S240/244). rpS6 and PKB were used as loading controls.

of as little as 0.2 μ M PF4708671 was sufficient to significantly inhibit exendin-4-stimulated DNA replication. Importantly, at this concentration, PF4708671 was unable to block the phosphorylation of rpS6, which is a primary target of S6K2 and not S6K1 (Pende *et al.* 2004; Fig. 2B). Therefore, PF4708671 is likely inhibiting DNA replication by inhibiting S6K1 rather than S6K2.

PKB is required for exendin-4 stimulation of both mTORC1 and islet cell DNA replication

As PKB is known to activate mTORC1 in other cell types via the phosphorylation and inhibition of TSC2 and the phosphorylation and dissociation of PRAS40 from mTORC1 (Kovacina *et al.* 2003, Oshiro *et al.* 2007, Sancak *et al.* 2007, Thedieck *et al.* 2007, Vander Haar *et al.* 2007, Wang *et al.* 2007), we determined whether exendin-4 activates mTORC1 and DNA replication through a PKB-dependent mechanism. We initially investigated the potential role of PKB in exendin-4 stimulation of β -cell DNA replication. Dispersed rat islets of Langerhans were cultured in 2 mM glucose, 7.8 mM glucose, or 7.8 mM glucose plus exendin-4 for 96 h and the incorporation of [3 H]-thymidine into DNA was determined in the presence or absence of AKTi1/2, a highly selective inhibitor of PKB (Barnett *et al.* 2005; Fig. 3A). Compared with cells incubated in 2 mM glucose, 7.8 mM glucose stimulated DNA replication and this was significantly potentiated by exendin-4. The addition of AKTi1/2 significantly inhibited exendin-4 plus glucose-stimulated DNA replication. Taken together, these data provide evidence that PKB activation is essential for GLP1R agonist-stimulated proliferation. Islets of Langerhans were then treated with exendin-4 plus glucose in the presence or absence of AKTi1/2, and changes in the phosphorylation/activation of PKB and mTORC1 were determined by western blotting analysis (Fig. 3B). Exendin-4 led to robust activation of PKB, as determined by its phosphorylation on S473, and the phosphorylation of its downstream targets GSK3, TSC2, and PRAS40. Importantly, an increase in the phosphorylation of TSC2 on T1462 and of PRAS40 on T246 were coincident with the activation of mTORC1, as determined by an increase in its auto-phosphorylation of S2481 and the phosphorylation of its downstream targets: S6K1 on T389 and rpS6 on S240/244 (Kovacina *et al.* 2003, Oshiro *et al.* 2007, Sancak *et al.* 2007, Thedieck *et al.* 2007, Vander Haar *et al.* 2007, Wang *et al.* 2007). The addition of AKTi1/2 inhibited exendin-4-stimulated phosphorylation of PKB, the phosphorylation of its downstream targets, and inhibited mTORC1 activation. Therefore, exendin-4-dependent mTORC1

Figure 3

GLP1R agonists activate mTORC1 and stimulate islet cell proliferation via a PKB-dependent mechanism. (A) Dispersed rat islets of Langerhans were incubated in RPMI containing 2 mM glucose, 7.8 mM glucose or 7.8 mM glucose plus 10 nM exendin-4 for 96 h. [3 H]-thymidine and, where indicated, AKTi1/2 were added during the last 24 h. DNA was extracted by TCA precipitation and [3 H]-thymidine incorporation was determined using a scintillation counter. *** P < 0.001. (B) Rat islets of Langerhans were incubated in either 2 mM glucose or 7.8 mM glucose plus exendin-4 (10 nM) in the presence or absence of 1 μ M AKTi1/2. Lysates were then analyzed by western blotting using specific antibodies against phospho (P)-PKB (S473), P-rpS6 (S240/244), P-PRAS40 (T246), P-S6K1 (T389), P-TSC2 (T1462), P-GSK3 (S9/21), P-mTOR (S2481), and total rpS6.

activation is likely mediated by the PKB-dependent phosphorylation of TSC2 and PRAS40.

Evidence that exendin-4 activates PKB/mTORC1 independently of EGFR transactivation

Given the critical importance of PKB in both exendin-4-stimulated mTORC1 activation and DNA replication,

we next investigated how exendin-4 activates PKB. As it has been reported that GLP1 acutely activates PI3K and stimulates the activation of FOXO1, a downstream target of PKB, via EGFR transactivation in the rat clonal pancreatic β -cell line INS (832/13; Buteau *et al.* 2003), we initially sought to confirm the role for EGFR transactivation in PKB-dependent mTORC1 activation. INS1E cells were pre-incubated with increasing doses of a selective EGFR inhibitor AG1478 (Levitzki & Gazit 1995) before treatment with GLP1 plus glucose or, as control, EGF (Fig. 4A and B). EGF-dependent ERK1/2 activation was effectively blocked by 2.5 nM AG1478 (Fig. 4B); yet AG1478 had no effect on GLP1-stimulated PKB phosphorylation or the activation of

mTORC1, as determined by the phosphorylation state of rpS6 (Fig. 4A). Similar results were obtained in the mouse clonal pancreatic β -cell line MIN6 (data not shown). To provide evidence that the results obtained in these β -cell lines were applicable to primary β -cells, rat islets of Langerhans were treated with glucose, exendin-4 plus glucose, or EGF in the presence or absence of 5 nM AG1478. EGF stimulated the phosphorylation of PKB and rpS6 and this was effectively blocked by 5 nM AG1478 (Fig. 4C). Yet AG1478 had no significant inhibitory effect on glucose or glucose plus exendin-4-stimulated PKB or rpS6 phosphorylation. Together, these data provide evidence that EGFR transactivation is not required for the activation of the PKB–mTORC1 pathway in response to GLP1R agonists in rat islets, INS1E cells, or MIN6 cells.

Evidence that exendin-4 activates PKB/mTORC1 via the autocrine/paracrine activation of the IGF1R

Another possible mechanism by which exendin-4 activates PKB is via the autocrine/paracrine action of, for example, insulin or IGF. To investigate this, islets of Langerhans were incubated with exendin-4 plus glucose in the presence or absence of diazoxide, a K^+ ATP channel opener, which inhibits exocytosis. As anticipated, diazoxide inhibited glucose or exendin-4 stimulated insulin secretion and this correlated with the inhibition of both PKB and rpS6 phosphorylation (Fig. 5A). To determine the potential role of insulin or IGF in exendin-4-stimulated PKB activation, InsR or IGF1R expression was knocked down using siRNA (Fig. 5B). Knockdown of InsR expression had no significant effect on exendin-4-stimulated PKB phosphorylation. In contrast, knockdown of IGF1R expression effectively inhibited exendin-4 and glucose-stimulated PKB and rpS6 phosphorylation. Together, these results provide evidence that exendin-4 activates PKB/mTORC1 via the autocrine/paracrine activation of the IGF1R in rat islets cells.

Figure 4

GLP1R agonist stimulated PKB and mTORC1 activation is independent of EGFR activation. (A) INS1E cells incubated in 2 mM glucose (Glu.) (control or C), where indicated, were preincubated for 30 min with AG1478 before treatment for 1 h with 7.8 mM Glu. plus 10 nM GLP1. (B) INS1E cells incubated in 2 mM Glu. (C), where indicated, were pretreated with AG1478 for 30 min before treatment for 5 min with EGF (20 ng/ml). (C) Rat islets of Langerhans incubated in 2 mM Glu. (control or C), where indicated, were treated for 30 min with AG1478 (5 nM) before incubation with 7.8 mM Glu. or 7.8 mM Glu. plus 10 nM exendin-4 (Glu. + Ex.) or EGF (20 ng/ml). In all cases, cells were lysed and proteins separated by SDS–PAGE, western blotted, and probed with antisera against P-ERK1/2, rpS6, ERK2, P-PKB (S473), P-rpS6 (S240/244), PKB, and GAPDH as indicated. All images shown are representative of three independent experiments.

Discussion

PKB and mTORC1 play important roles in the regulation of many cellular processes such as growth, survival, proliferation, and metabolism. In this report, we provide evidence that exendin-4 potentiates glucose-stimulated PKB activation in β -cells via the autocrine/paracrine activation of the IGF1R. We also provide evidence that this is necessary for GLP1-stimulated islet cell proliferation and that this is likely mediated through the activation of mTORC1. Moreover, we show that the positive effect of

Figure 5

Exendin-4 (Ex.4) activates PKB and mTORC1 through the autocrine or paracrine activation of the IGF1R. (A) Rat islets of Langerhans were incubated in either 2 mM glucose (Glu.) (control or C) 7.8 mM Glu. or 7.8 mM plus 10 nM Ex.4 (Glu. + Ex.) in the presence or absence of 250 μ M diazoxide for 30 min. An insulin ELISA assay was carried out on the media. The cell lysates were analyzed by western blotting using anti-phospho (P)-PKB (S473), P-GSK3 (S9/21), P-rpS6 (S240/244), and anti-PKB. (B) Dispersed rat islets of Langerhans were transfected with siRNA directed against the β -subunit of the insulin receptor (InsR) or the IGF1R for 48 h. The cells were then incubated in either 7.8 mM Glu. or 7.8 mM Glu. plus 10 nM Ex.4 (Glu. + Ex.) for 30 min. The cell lysates were analyzed by western blotting for phospho (P)-PKB (S473), P-rpS6 (S240/244), InsR β -subunit, IGF1R β -subunit and rpS6. All western blots shown are representative of three independent experiments.

exendin-4 on proliferation requires the activation of S6K1, a downstream target of mTORC1.

Buteau *et al.* (2003) reported that GLP1 stimulates PI3K via the transactivation of the EGFR in the pancreatic β -cell line INS (832/13). Interestingly, in this report, we show that EGFR transactivation is unlikely to be responsible for GLP1R-stimulated PI3K activation as AG1478 had no effect on exendin-4-stimulated PKB phosphorylation in either clonal pancreatic β -cell lines (i.e. INS1E (this report) and MIN6 (N M El Sayed, C E Moore and T P Herbert, unpublished results)) or rat islets of Langerhans at concentrations that effectively block EGF-induced PKB or ERK1/2 phosphorylation (Fig. 4). One potential reason for these differences could simply be due to the different cell lines used. Alternatively it could be due to differences in

the concentration of AG1478 used. Buteau *et al.* (2003) used 250 nM of AG1478 to block PI3K activity, whereas we show that 2.5 nM is sufficient to block EGFR signalling to ERK1/2 and PKB (Fig. 4). Indeed, we have found that 250 nM AG1478 also inhibits insulin-stimulated PKB phosphorylation (N M E S, C E M and T P H, unpublished results), demonstrating that this inhibitor at this concentration is not specific for the EGFR.

Chronic treatment of pancreatic β -cells with GLP1 stimulates increased IGF1R expression and the release of IGF2 from the pancreatic β -cell, which in turn activates PKB (Cornu *et al.* 2009, 2010), presumably via a PI3K-dependent mechanism. It is therefore possible that acute treatment of islets with exendin-4 may also potentiates glucose-stimulated IGF2 secretion and activates the PI3K/PKB via the autocrine/paracrine activation of the IGF1R (Fig. 5), although in these studies we have not measured IGF2 release. However, there are a number of other potential endogenous agonists for the IGF1R, which are released by the β -cell, including its high-affinity agonist IGF1 and insulin, which although binds at much lower affinity, is likely to be present at much higher

Figure 6

Schematic representation of the signalling pathways leading to GLP1R agonist stimulation of islet cell proliferation investigated in this study. (1) GLP1 receptor activation results in the activation of the heterotrimeric stimulatory G-protein (Gs) which activates adenyl cyclase (AC). (2) The activation of AC leads to the potentiation of glucose-stimulated IGF secretion. (3) IGF/insulin binds to the IGF1R (4) and stimulates the PI3K/PKB pathway leading to the activation of mTORC1, which in turns activates S6K1. (5) S6K1 stimulates islet cell proliferation.

concentrations (Van Schravendijk *et al.* 1987, Zhang *et al.* 1997, Pandini *et al.* 2002). Thus, it is also equally possible that these may activate the IGF1R in response to GLP1R activation. Transgenic mouse studies have provided evidence that the IGF1R plays an important positive role in pancreatic β -cell function, as β -cell-specific IGF1R knockout (*IGF1R*^{-/-}) mice have glucose intolerance and altered glucose sensing (Kulkarni *et al.* 2002). However, in contrast to β -cell-specific InsR knockout mice, adult β -cell mass is not compromised in β -cell specific IGF1R knockout mice (Kulkarni 2002, 2005, Kulkarni *et al.* 2002, 2004). Interestingly, studies on inter-crossed IGF1R and insulin receptor substrate 2 (*IRS2*; *IGF1R*^{+/-}*IRS2*^{+/-}) heterozygote knockout mice revealed that insulin resistance induced increases in β -cell mass and islet development during mediation of embryogenesis by the autocrine/paracrine activation of the IGF1R through coupling to *IRS2* (Withers *et al.* 1999). It would be helpful to know, whether *in vivo* administration of GLP1 or exendin-4 can enhance murine β -cell proliferation, and if it does, whether this proliferative effect is abolished in *IGF1R*^{-/-} mice, yet this is beyond the scope of this study. In INS1 cells, GLP1 was also shown to increase the level of tyrosine phosphorylation on *IRS2*, which co-immunoprecipitates with the p85 regulatory subunit of PI3K (Trumper *et al.* 2000). As we provide evidence that exendin-4 activates the PI3K/PKB pathway via the autocrine/paracrine activation of the IGF1R, we suggest that the most likely mode of activation of PI3K by GLP1R agonists is via *IRS2* (Figs 5 and 6).

In this report, we also show that GLP1R agonists are able to enhance β -cell replication via an mTORC1-dependent pathway and that this is likely mediated by PKB-dependent phosphorylation and inactivation of TSC2 (Inoki *et al.* 2002, Potter *et al.* 2002; Fig. 3) and the phosphorylation of PRAS40, which results in its dissociation from mTORC1 and permits the recruitment of S6K and 4EBP to mTORC1 (Kovacina *et al.* 2003, Oshiro *et al.* 2007, Sancak *et al.* 2007, Thedieck *et al.* 2007, Vander Haar *et al.* 2007, Wang *et al.* 2007). Indeed, these observations concur with transgenic mouse studies show that hyperactivation of mTORC1 or PKB stimulates an increase in β -cell mass/proliferation *in vivo* (Xie & Herbert 2012). These effects may be mediated via the mTORC1-dependent activation of *CDK4*, and an increase in cyclin D₂ and D₃ (Balcazar *et al.* 2009), possibly mediated by a decrease in cap-dependent translation via the hypo-phosphorylation of 4EBPs (Beretta *et al.* 1996, Gingras *et al.* 1998). However, glucose and exendin-4 only modestly stimulates 4EBP1 phosphorylation in isolated

islets (Fig. 1). Moreover, 4EBP knockout mice have been generated and characterised but no effects on β -cell mass or glucose homeostasis have been reported (Tsukiyama-Kohara *et al.* 2001, Le Bacquer *et al.* 2007). Interestingly, we show that inhibition of another target of mTORC1, S6K1, inhibits islet cell proliferation. This is perhaps a little unexpected, as although S6K1 knockout (*S6K1*^{-/-}) mice have reduced β -cell mass, this has been reported to be due to a reduction in β -cell size (Pende *et al.* 2000).

In summary, GLP1R agonists stimulate β -cell proliferation via an mTORC1/S6K1-dependent pathway whose activation is mediated through the autocrine/paracrine activation of the IGF1R. This work provides a better understanding of the molecular basis of GLP1 agonist-induced β -cell proliferation and could be exploited in the identification of novel compounds for their ability to stimulate β -cell regeneration and/or β -cell mass for the treatment of T1DM and/or T2DM.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

N M E S and J X made a significant contribution to the acquisition, analysis, and interpretation of the data. X Z, C Q, and C E M made a significant contribution to the acquisition of the data. T P H made a substantial contribution in the conception, design, analysis, and interpretation of data and wrote the article.

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