Exogenous expression of glucagon-like peptide 1 receptor and human insulin in AtT-20 corticotrophs confers cAMP-mediated gene transcription and insulin secretion

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Abstract

The insulinotropic effects of glucagon-like peptide 1 (GLP-1) are mediated by its seven-transmembrane receptor (GLP-1R) in pancreatic β -cells. We have transiently transfected the GLP-1R and a proopiomelanocortin (POMC) promoter-driven human preproinsulin gene vector (pIRES) into the AtT-20 pituitary corticotrophic cell line, to investigate the possibility of creating a regulated, insulin-expressing cell line. Receptor expression was confirmed by RT-PCR and functionality was demonstrated by measuring changes in cAMP levels in response to GLP-1. Rapid (5 min) stimulation of cAMP production was observed with 100 nM GLP-1, 24 h after transfection of 2 μ g GLP-1R DNA. AtT-20 cells co-transfected with GLP-1R and human glycoprotein hormone α -subunit or

rat POMC promoters revealed GLP-1-stimulated cAMP activation of transcription. Co-transfection of the pIRES vector with the GLP-1R resulted in GLP-1-stimulated activation of POMC promoter-driven preproinsulin gene transcription but insulin secretion was not detected. However, using an adenoviral expression system to infect AtT-20 cells with GLP-1R and the preproinsulin gene (including 120 bp of its own promoter) resulted in a 6.4 ± 0.6 -fold increase in cAMP and a 4.9 ± 0.8 -fold increase in insulin secretion in response to 100 nM GLP-1. These results demonstrate, for the first time, functional GLP-1R-mediated preproinsulin gene transcription and secretion in a transplantable cell line.

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Introduction

Glucagon-like peptide 1 (GLP-1) is a proglucagon-derived peptide hormone that is synthesized and secreted by intestinal L-cells in response to the ingestion of nutrients and circulates to the pancreas where it stimulates the synthesis and secretion of insulin in a glucose-dependent manner. Indeed, GLP-1 has turned out to be the most potent and efficacious regulatory stimulator of insulin secretion discovered so far (Fehmann *et al.* 1995). These beneficial effects have led to trials of GLP-1, and its more stable and long-acting analogues, as potential therapeutic agents for the treatment of type 2 diabetes (Holst 1999, Drucker 2002). An alternative approach to therapy with GLP-1 would be to engineer a glucose-sensitive surrogate insulin-secreting cell by exogenously transfecting the GLP-1 receptor into a transplantable cell line.

Previous work has shown that mouse anterior pituitary cells (AtT-20 cells), when transplanted under the adrenal capsule, failed to elicit an immune response from the host T lymphocytes (Lipes *et al.* 1996). Several attempts have been made to use these cells to engineer an insulin-

secreting transplantable cell line since they have a number of important similarities to pancreatic β -cells. Although they do not express insulin, AtT-20 cells do express the proinsulin processing endopeptidases, prohormone convertase 1 and 2 (PC1, PC2) enabling them correctly to convert and process transfected human preproinsulin cDNA to mature insulin (Moore *et al.* 1993). In addition, they contain a predominantly regulated pathway of secretion, rather than a constitutive pathway of secretion, enabling stored hormone to be released only on stimulus with the appropriate signal. However, they do not secrete insulin in response to glucose, and others have attempted to overcome this lack of glucose responsiveness by transfection of the pancreatic glucose transporter GLUT2 (Hughes *et al.* 1992, Davies *et al.* 1998). Disappointingly, GLUT2 transfected cells only released insulin in response to subphysiological increases in glucose concentration, as phosphorylation became the rate limiting step for generation of glucose generating signals, and the predominant glucose phosphorylating enzyme in pituitary cells is hexokinase, a low K_m (approx 10 μ M) enzyme which is saturated at very low glucose concentrations.

Generating glucose-stimulated insulin production by over expression of both GLUT2 and glucokinase led to premature cell death through glucose toxicity, generating an excess of hexose intermediates in the glycolytic pathway with attendant consumption of ATP (Faradji *et al.* 2001).

In the present study, we have taken an alternative approach by co-transfecting AtT-20 cells with the GLP-1 receptor (GLP-1R) and the human preproinsulin gene driven by the proopiomelanocortin (POMC) promoter. The POMC gene promoter contains a cAMP response element, which would allow it to respond to GLP-1-activated cAMP stimulation in AtT-20 cells, thus bypassing the limitations of phosphorylation of glucose intermediates. POMC-luciferase (POMC-LUC) and POMC-insulin-luciferase (POMCINS-LUC) reporter plasmids were co-transfected to investigate the GLP-1 responsiveness of the promoter and subsequent promoter-driven insulin gene transcription. We have also used an adenoviral expression system to investigate the effects of GLP-1 on cAMP generation and insulin secretion.

Materials and Methods

Materials

All reagents were purchased from Sigma Chemicals (Poole, Dorset, UK) unless otherwise stated. GLP-1 was diluted in sterile distilled water to form a stock solution of 1 mM and was stored at -20°C until diluted in culture medium. 3-Isobutylmethylxanthine (IBMX) was dissolved directly in culture medium to a final concentration of 1 mM. Forskolin (FSK) was dissolved in sterile dimethylsulphoxide (DMSO) to form a stock solution of 0.1 M and stored at 4°C until further dilution to 10 μM with culture medium. H-89, a protein kinase A (PKA) inhibitor, was purchased from Calbiochem (CN Biosciences Ltd, Nottingham, Notts, UK) and stored as a stock solution of 1 mM before dilution with culture medium to a final concentration of 1 μM . All stimulants were dissolved in culture medium directly before use.

Plasmids

The full-length rat GLP-1R cloned into pcDNA was a gift from Prof. B Thorens, (University of Lausanne, Switzerland). The coding region of the gene was amplified using specific primers (GenBank accession number NM_012728) GLP-1R forward 5'-CCCGCCATGGC CGTCACCCCCAGCCTG-3' and GLP-1R reverse 5'-GGCTCAGCTGCAGGAATTTTGGCAGGT-3' resulting in a PCR product 1400 bp in size. Amplification was carried out using the Advantage-GC2 PCR Kit (Clontech Laboratories Inc., Palo Alto, CA, USA) in a total reaction volume of 50 μl under the following conditions: 94°C for 30 s, 68°C for 60 s, 68°C for 60 s for

35 cycles. The resultant PCR product was visualized by 1% agarose gel electrophoresis with ethidium bromide staining. This product was sub-cloned into the EcoRI site of the ampicillin resistant expression vector pCR3.1 (Invitrogen, Groningen, The Netherlands), downstream of the cytomegalovirus immediate/early promoter (CMV). Plasmids were grown in JM109 sub-cloning efficiency grade *Escherichia coli* (Promega Corporation, Southampton, Hants, UK) and purified using the Qiagen Mega Kit method (Qiagen, Crawley, West Sussex, UK). DNA was quantitated using spectrophotometric absorbance values at A_{260} and the A_{260}/A_{280} ratios were between 1.7 and 1.9. The presence and correct orientation of the GLP-1R insert was confirmed by restriction enzyme analysis and subsequent agarose gel electrophoresis. Product identity was confirmed using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, UK) for direct automated DNA sequencing.

The POMC-LUC reporter plasmid was a gift from Dr Malcolm Low (University of Chicago, IL, USA) and consists of a 780 bp region of the POMC promoter (-706 bp to $+64$ bp) cloned into the pGEM7+ vector, upstream of the firefly luciferase gene (Low 1993). The reporter plasmid $\alpha\text{GSU-LUC}$ contains 517 bp of the 5' flanking sequence and 44 bp of exon 1 of the human glycoprotein hormone α -subunit (αGSU) promoter linked to the luciferase gene in the pA3 LUC plasmid (Holdstock *et al.* 1996). The Bos β Gal reporter plasmid contains the promoter of the human elongation factor 1 gene driving β -galactosidase expression. Cellular extracts were assayed for luciferase and β -galactosidase as previously described (Burrin *et al.* 1998).

The POMCINS-LUC plasmid was constructed as follows. Full-length human insulin genomic clone Phins 300 cloned into vector pBR327 was a gift from Prof. G Bell (Chicago, IL, USA). Specific primers were designed: INS forward 5'-AGGGTGAGCCAACCGCCCATTTG-3', INS reverse 5'-CTAGGTTCTCGTCTGCCCACTA-3' to amplify a 1660 bp fragment, using the Expand High Fidelity PCR System (Roche Diagnostics GmbH, Mannheim, Germany), under the following conditions: 94°C for 120 s, 40 cycles of 94°C for 30 s, 65°C – 55°C for 30 s (10 cycles), 68°C for 180 s. Following sub-cloning, the amplified preproinsulin gene and the POMC promoter from the POMC-LUC plasmid were cloned into the NotI and EcoRI sites of the pIRESneo plasmid (Clontech Laboratories). The luciferase gene from the pGL3 Basic reporter plasmid (Promega) was cloned into the SmaI and XbaI sites replacing the neomycin resistance gene of the p internal ribosome entry site (IRES) neo plasmid. Plasmids were sequenced, purified and quantitated as described above for the GLP-1 expression vector.

PCR-amplified GLP-1R DNA (1400 bp) and preproinsulin DNA (1748 bp, including 120 bp of the 5' flanking sequence) were cloned into the NotI/SalI sites

of the pShuttle CMV vector (Stratagene, La Jolla, CA, USA). Preparation and recombination of these vectors into the pAdEasy vector (Stratagene) was performed as described previously (He *et al.* 1998). AtT-20 cells were infected with the GLP-1R (pAdGLP-1R) and/or the preproinsulin gene (pAdIns). Empty adenovirus vector was used as control. Viral titre was determined using a plaque assay and represented as multiplicity of infection (MOI) as described by He *et al.* (1998).

Cell culture

AtT-20 cells were maintained in 75-cm² culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) containing 2 mM L-glutamine and 25 mM glucose, and supplemented with 10% (v/v) horse serum (HS) (Life Technologies, Paisley, Strathclyde, UK), 100 U/ml penicillin-G and 10 mg/ml streptomycin sulphate at 37 °C, in a 5% CO₂-95% air atmosphere. For cAMP measurement, cells were seeded at 1×10^6 cells per well in 6-well tissue culture plates 24 h prior to transfection. For luciferase measurement, cells were plated at a density of 3×10^5 cells per well in 24-well tissue culture plates 24 h prior to transfection.

Transient transfections of AtT-20 cells

AtT-20 cells were transfected by the calcium phosphate technique without glycerol shock with either 2–20 µg pCRGLP-1R DNA, or the following plasmids: 2 µg pCR3.1 empty vector DNA, 1–20 µg POMCINS-LUC, 1.5 µg αGSU-LUC or 1.5 µg BosβGal, which served as an internal control to normalize for transfection efficiency. Cells were stimulated for 5 min to 8 h with 1 µM–1 nM GLP-1 or 10 µM FSK in 500 µl culture medium. All stimulations for cAMP experiments were performed in the presence of 1 mM of the phosphodiesterase inhibitor, IBMX, and cells were pre-treated with or without 1 µM of the PKA inhibitor H-89 for 30 min prior to addition of GLP-1.

cAMP and insulin enzyme immunoassay (EIA)

cAMP levels were determined according to the manufacturer's instructions for acetylated samples using the Biotrak cAMP enzyme immunoassay kit (Amersham Pharmacia Biotech UK Ltd). Transfected cells were stimulated as described, and the reaction halted with the addition of 500 µl 100% ethanol pre-chilled to –20 °C; the cells were frozen at –20 °C overnight before thawing and pelleting by centrifugation at $500 \times g$, for 5 min at 4 °C. The resulting supernatant was collected and vacuum dried and the pellets re-suspended in 500 µl of the assay buffer provided. Once re-suspended, the samples were pelleted by centrifugation, as before, and the supernatant removed and stored at –70 °C until assayed (intra-assay

coefficient of variation (CV) of 4.3–6.3%, interassay CV of 8.2–17.4%). Insulin secretion in the media of cultured cells was measured using the Biosource human insulin enzyme immunoassay kit (Biosource Europe SA, Nivelles, Belgium). The intra- and interassay coefficients of variation were 5.3% and 5.6% respectively at an insulin concentration of 17.5 mU/l.

Reverse transcription-PCR analysis

In separate experiments, mRNA was isolated from transfected AtT-20 cells using the QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech, UK) using oligo (deoxythymidine)-cellulose washed with high and low salt buffers and elution of mRNA in 400 µl elution buffer. Quantitation was performed by measurement of absorbance at 260 nm, and ranged between 0.7 and 3.4 µg mRNA from 1×10^6 cells. mRNA was reverse transcribed using the First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, UK), with a Not 1-(deoxythymidine)₁₈ bifunctional primer in a reaction catalysed by the Moloney murine leukaemia virus reverse transcriptase. RT-PCR analysis was performed in a total reaction volume of 50 µl using the GLP-1R and insulin gene specific intron spanning primers, as described above. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified, using identical conditions, as a control for the mRNA integrity of each sample using specific intron spanning primers (GenBank accession number NM_008084) GAPDH forward 5'-TGCACCACCAAC TGCTTAG-3', GAPDH reverse 5'-CCACCACCCTG TTGCTGTAG-3'. Negative controls, with water instead of cDNA template, were included in the RT-PCR amplification. All products were visualized by 1% agarose gel electrophoresis with ethidium bromide staining and the identity of the products confirmed by direct DNA sequencing.

Scanning densitometry

Scanning densitometry analyses were performed on RT-PCR products using the Kodak 1D 3.5 imaging software (Eastman Kodak Company, Scientific Imaging Systems, New York, USA). Band intensity values for GLP-1R were normalized to those for GAPDH and the resultant GLP-1R:GAPDH ratios were plotted to illustrate variations in gene expression.

Statistical analysis

All graphical data were plotted using GraphPad Prism 3.0 software (GraphPad, San Diego, CA, USA). Data presented are normalized and pooled from several experiments with typically three and four replicates per group, or presented as results that are representative of multiple experiments. Statistical analyses were performed using the

Figure 1 Transient expression of GLP-1R in AtT-20 cells. (A) GLP-1-stimulated cAMP accumulation in AtT-20 cells transiently transfected with GLP-1R (0 to 20 μg) and cultured for 24 h. Cells were stimulated with 100 nM GLP-1 in the presence of 1 mM IBMX for 15 min. Data shown are representative of at least 3 independent experiments, normalized as fold increase over basal. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from basal. (B) Representative gel showing GLP-1R and GAPDH expression by RT-PCR from transiently transfected AtT-20 cells (0 or 2 μg DNA/well) and cultured for up to 72 h. Data shown are representative of 3 independent experiments. (C) Short time course of GLP-1-stimulated cAMP accumulation in transiently transfected AtT-20 cells. Cells were transfected with 2 μg GLP-1R, cultured for 24 h then stimulated with 0 or 100 nM GLP-1 in the presence of IBMX for up to 30 min. Data shown are pooled from 3 independent experiments in triplicate, normalized as fold increase over basal for each time point. * $P < 0.05$, *** $P < 0.001$, significantly different from basal. (D) Dose-response of GLP-1-stimulated cAMP accumulation in transiently transfected AtT-20 cells. Cells were transfected with 2 μg pCR3.1 or GLP-1R and cultured for 24 h before stimulation with 100 nM GLP-1 in the presence of 1 mM IBMX for 15 min. Data shown are pooled from 3 independent experiments in triplicate, normalized as fold increase over empty vector (pCR3.1), shown as horizontal dashed line, for each time point. ** $P < 0.01$, significantly different from basal.

statistical tools provided as part of the GraphPad package, and data were analysed by ANOVA followed by Tukey's multiple comparison post-test or Student's *t*-test.

Results

Characterization of AtT-20 cells transfected with GLP-1 receptor

Increasing amounts (0.5–20 μg) of GLP-1 receptor DNA were transfected into AtT-20 cells to establish the optimum amount of DNA required for maximum cAMP

production. Initial RT-PCR analysis of transfected cells failed to detect PCR products following transfection of 0.5 and 1.0 μg GLP-1R DNA (data not shown). Subsequent experiments were therefore performed using 2.0, 5.0, 10.0 and 20.0 μg DNA. Intracellular cAMP accumulation in the presence of IBMX was measured following stimulation with 100 nM GLP-1 for 15 min to characterize the transfected AtT-20 cells (Fig. 1A). The initial concentration of GLP-1 used (100 nM) and the duration of the stimulation (15 min) were chosen from previously published data (Montrose-Rafizadeh *et al.* 1999). Maximal cAMP accumulation was observed 24 h after transfection

of 2.0 µg DNA with a 4.9 ± 1.1 -fold increase above basal ($P < 0.001$). The GLP-1-stimulated cAMP response decreased when further amounts of DNA were transfected, although significant stimulation of cAMP production also resulted from transfection of 5 µg and 10 µg DNA with fold increases above basal of 3.3 ± 0.3 ($P < 0.001$) and 2.3 ± 0.3 ($P < 0.01$) respectively. Importantly, no cAMP response to GLP-1 was seen in cells transfected with the empty vector implying that the observed cAMP response was mediated by the transfected GLP-1 receptor (data not shown).

Having established that 2 µg GLP-1R DNA was sufficient for maximal cAMP responsiveness, time course experiments were performed to establish the optimal time post-transfection at which to stimulate the cells. Both RT-PCR and cAMP assays were used to characterize the response to stimulation with 100 nM GLP-1 for 15 min in the presence of IBMX at different times post-transfection. RT-PCR analysis confirmed that the GLP-1R is not endogenously expressed in AtT-20 cells (Fig. 1B) but did demonstrate the appearance of GLP-1R mRNA 12 h post-transfection. Expression of GLP-1R was maintained for 72 h post-transfection (Fig. 1B). cAMP assay data agreed with the RT-PCR analysis showing a similar significant 4.1 ± 0.1 -, 4.7 ± 0.2 - and 4.6 ± 0.2 -fold increase in GLP-1-stimulated cAMP levels post-transfection at 24, 48 and 72 h respectively (all $P < 0.001$). Based on these data, subsequent experiments were conducted 24 h post-transfection.

To investigate the effects of GLP-1 stimulation on cAMP accumulation, cells were transfected with 2 µg GLP-1R and stimulated for 5, 15 or 30 min in medium with or without 100 nM GLP-1 in the presence of IBMX. A significant and sustained accumulation in cAMP levels of three- to fourfold was observed for all time points studied (Fig. 1C). Following 5-min stimulation, cAMP levels were 4.0 ± 0.5 -fold ($P < 0.001$) higher than basal levels. No additional generation of cAMP was detected after 5 min. As all periods between 5 and 30 min of stimulation showed similar significant increases in cAMP accumulation, further experiments were performed with 15 min GLP-1 stimulation.

To determine the optimum dose of GLP-1 stimulation, GLP-1R (2 µg)-transfected AtT-20 cells were stimulated 24 h post-transfection with concentrations of GLP-1 ranging from 1 nM to 1 µM for 15 min in the presence of IBMX, and cAMP production was measured (Fig. 1D). The results showed a dose-dependent increase in cAMP production above basal levels from 1 nM to 100 nM GLP-1, with the greatest fold increase of 3.7 ± 1.0 ($P < 0.01$) above basal arising following stimulation with 100 nM GLP-1.

GLP-1R transfection of AtT20 cells confers cAMP-mediated gene transcription

To confirm that GLP-1-stimulated cAMP production elicited a transcriptional response, two pituitary gene

promoters (α GSU-LUC and POMC-LUC), which both contain known cAMP-response elements (CREs), were transfected into AtT-20 cells. Cells transfected with α GSU-LUC were stimulated with 100 nM GLP-1 for 8 h before the luciferase activity was ascertained (Fig. 2A). GLP-1 stimulated α GSU promoter activity 13.9 ± 1.7 -fold ($P < 0.001$) above basal promoter activity, with FSK (1 µM) also inducing a significant response of 8.1 ± 1.0 -fold ($P < 0.01$) above basal. When the POMC promoter was transfected into the cells, GLP-1 stimulation induced a modest but significant 2.2 ± 0.3 -fold ($P < 0.05$) increase in promoter activity above basal levels (Fig. 2B). Forskolin failed to produce a significant activation of the POMC promoter.

In order to demonstrate the involvement of PKA in GLP-1-stimulated promoter activity, cells transfected with the α GSU-LUC plasmid were pre-treated with 1 mM of the PKA inhibitor, H-89, prior to stimulation with 100 nM GLP-1 for 8 h (Fig. 2C). Inhibitor pretreatment resulted in a significant decrease in α GSU promoter activity in response to GLP-1 stimulation, with a decrease from 14.9 ± 0.5 -fold above unstimulated cells to 9.4 ± 1 -fold ($P < 0.01$) in H-89-pretreated cells. A similar reduction in FSK-stimulated α GSU promoter activity from 11.9 ± 2.6 -fold to 5.1 ± 0.5 -fold ($P < 0.01$) was also seen with H-89 pretreatment of transfected cells (Fig. 2C). These results suggest that PKA activation is partially mediating the transcriptional response to GLP-1 stimulation in AtT-20 cells transfected with the GLP-1R.

Having shown transfected GLP-1R to be functional in AtT-20 cells, the GLP-1 responsiveness of the transfected preproinsulin gene was investigated. Cells were transfected with different amounts (1–20 µg) of the POMCINS-LUC plasmid. RT-PCR revealed insulin mRNA expression with all amounts transfected (Fig. 3A) and scanning densitometry analysis suggested maximal expression occurred with 5 µg transfected DNA. To investigate the transcriptional response elicited by GLP-1 stimulation of the transfected insulin gene, cells co-transfected with GLP-1R and POMCINS-LUC were stimulated with 100 nM GLP-1 for 8 h (Fig. 3B). The luciferase activity showed a significant increase in cells co-transfected with the receptor and the preproinsulin gene in response to GLP-1 of 3.4 ± 0.6 -fold over basal ($P < 0.001$). Significantly increased luciferase activity of 4.0 ± 0.5 -fold above basal was also achieved with FSK stimulation ($P < 0.001$). These data suggest that the preproinsulin gene, when driven by the POMC promoter, is both GLP-1 and cAMP responsive. However, using this transient expression system, we were unable to detect insulin secretion into the media of cells co-transfected with GLP-1R and POMCINS-LUC and stimulated with GLP-1. To investigate whether this was due to low transfection efficiency or absence of insulin gene translation, we used an adenoviral expression system.

Initially, we investigated cAMP accumulation in AtT-20 cells infected with pAdGLP-1R (10–200 MOI) or empty

Figure 3 POMCINS expression: characterization and GLP-1 responsiveness in transfected AtT-20 cells. (A) Gel showing insulin and GAPDH expression by RT-PCR in AtT-20 cells transfected with the indicated concentrations of POMCINS plasmid DNA. (B) Luciferase activity was analysed as a measure of insulin gene transcription following transient transfection of AtT-20 cells with 5 μg POMCINS and 5 μg BosβGal and cultured for 24 h. Cells were subsequently stimulated with 0 or 100 nM GLP-1 or 10 μM FSK for 8 h. Data shown are pooled from 3 independent experiments, expressed as fold increase over basal. *** $P < 0.001$, significantly different from basal.

Figure 2 GLP-1-dependent gene transcription in transfected AtT-20 cells. AtT-20 cells were transiently transfected with GLP-1R (2.0 μg) and BosβGal (1.5 μg), and with α GSU-LUC or POMC-LUC (1.5 μg), and cultured for 24 h. (A) α GSU-LUC/GLP-1R-transfected AtT-20 cells were stimulated for 8 h with 0 or 100 nM GLP-1 or 10 μM forskolin (FSK). Data shown are pooled from 2 independent experiments in quadruplicate, expressed as fold increase over basal. ** $P < 0.01$, *** $P < 0.001$, significantly different from basal promoter activity. (B) POMC-LUC/GLP-1R-transfected AtT-20 cells were stimulated for 8 h with 0 or 100 nM GLP-1 or 10 μM FSK. Data shown are pooled from 2 independent experiments in quadruplicate, expressed as fold increase over basal. * $P < 0.05$, significantly different from basal promoter activity. (C) α GSU-LUC/GLP-1R-transfected AtT-20 cells were pretreated with 0 or 1 μM H-89 for 30 min prior to stimulation with 0 or 100 nM GLP-1 or 10 μM FSK for 8 h. Data shown are pooled from 2 independent experiments in quadruplicate, expressed as fold increase over basal. * $P < 0.05$, ** $P < 0.01$, significantly different between control and H89 treatment. *** $P < 0.001$, significantly different from basal promoter activity.

vector (pAd), cultured for 24 h and subsequently stimulated for 15 min with 100 nM GLP-1 in the presence of 1 mM IBMX. A significant increase in cAMP generation was observed with all concentrations of pAdGLP-1R, reaching a peak of 6.4 ± 0.6 -fold increase over empty vector with 50 MOI pAdGLP-1R (Fig. 4A). Similar significant increases were seen with 20 and 100 MOI pAdGLP-1R. Co-infection of pAdGLP-1R (20 MOI) and pAdIns (50 MOI) for 24 h followed by 15 min stimulation with 100 nM GLP-1 resulted in a threefold increase in insulin concentration in the media, achieving a level of 27.9 ± 6.0 mU/l (Fig. 4B). Importantly, no increase in insulin release was seen when pAdIns was infected in the absence of pAdGLP-1R, nor was there an insulin response to empty vector (pAd) or the pAdGLP-1R in the absence of pAdIns (Fig. 4B). In separate experiments, altering the amount of pAdIns used to infect the cells from 10 to 200 MOI demonstrated a dose-dependent increase from 10 to 50 MOI with a maximum 4.9 ± 0.8 -fold increase to achieve an insulin concentration of 39.9 ± 2.9 mU/l with 50 MOI pAdIns (Fig. 4C).

Discussion

In this study we have used the pituitary corticotroph AtT-20 cell line to create a GLP-1 responsive cell and demonstrated GLP-1-mediated POMC promoter-driven preproinsulin gene transcription and insulin secretion. Transfection of the cells with the GLP-1R conferred GLP-1 responsiveness to a cell line that does not normally express the receptor. RT-PCR analysis of untransfected AtT-20 cells confirmed the absence of endogenous GLP-1R in pituitary corticotroph cells suggesting that, as previously observed (Satoh *et al.* 2000), cells other than corticotrophs, possibly thyrotrophs, are responsible for GLP-1R immunoreactivity in the anterior pituitary.

Figure 4 Adenoviral infection of pAdGLP-1R and pAdIns in AtT-20 cells. (A) AtT-20 cells were infected with pAdGLP-1R (10–200 MOI) or empty vector (pAd) and cultured for 24 h. Cells were stimulated with 100 nM GLP-1 in the presence of 1 mM IBMX for 15 min. cAMP accumulation is shown as fold increase above pAd. Data shown are pooled from 2 independent experiments each performed in triplicate. *** $P < 0.001$, significantly different from pAd. (B) AtT-20 cells were infected with empty vector (pAd) or pAdIns (50 MOI) and/or pAdGLP-1R (20 MOI) and cultured for 24 h. Cells were then incubated for 15 min in the absence or presence of 100 nM GLP-1. Insulin secretion data (mU/l) are pooled from 2 independent experiments each performed in triplicate. *** $P < 0.001$, significantly different from pAd. (C) AtT-20 cells were infected with either empty vector (pAdEasy) or pAdIns (10–200 MOI) and pAdGLP-1R (20 MOI) and cultured for 24 h. Cells were then incubated for 15 min with 100 nM GLP-1. Insulin secretion data (mU/l) are pooled from 2 independent experiments each performed in triplicate and expressed as fold increase over empty vector. *** $P < 0.001$, significantly different from pAdEasy.

Our study showed that increasing the amount of receptor DNA transfected into the cells above 2 µg, resulted in a decrease in cAMP levels until transfection of 20 µg DNA failed to increase cAMP production above that in untransfected cells. This decrease in cAMP production could be a result of homologous or heterologous receptor desensitization with increased expression of transfected receptor. Use of another GLP-1R-negative cell line, the Chinese hamster ovary cells (CHO), demonstrated that cells expressing the greatest number of transfected GLP-1Rs exhibited desensitization (Fehmann *et al.* 1998). This suggests that a threshold for functional receptor expression exists. In transfected monkey kidney (COS-7) cells, both GLP-1 and the phorbol ester, phorbol 12-myristate 13-acetate are able to induce receptor phosphorylation and desensitization (Widmann *et al.* 1997). This suggests that transfection of 20 µg receptor DNA may result in GLP-1-mediated, cAMP-induced receptor phosphorylation and desensitization in AtT-20 cells.

Our study showed that transfected receptors in AtT-20 cells respond to GLP-1 at concentrations ranging from 1 nM to 1 µM, comparable to those shown previously in CHO cells, which achieved significant cAMP production with GLP-1 at 1 nM and 10 nM (Montrose-Rafizadeh *et al.* 1999). COS-7 cells responded to GLP-1 concentrations ranging from 1 nM to 1 µM with maximal cAMP production in response to 1 µM (Wheeler *et al.* 1993). In a separate study, the same cells exhibited a cAMP fold increase of 2.5 above basal in response to 100 nM GLP-1 (Dillon *et al.* 1993), similar to the fourfold increase seen in our cells.

The cAMP responses achieved as a result of GLP-1 stimulation post transfection with the receptor in this study demonstrated the presence of a functional receptor. Whether the cAMP response obtained was of a sufficient magnitude to activate gene transcription required investigation. To address this question, AtT-20 cells were transfected with known cAMP responsive promoters, the endogenous POMC and the exogenous αGSU promoters. Activation of the POMC promoter with GLP-1 stimulation would demonstrate a functional transcriptional system in AtT-20 cells, which might then be adapted for insulin gene transcription. The transfected region of the POMC promoter used in this study possesses a cAMP inducible element within the first exon, which is responsive to hypophysiostrophic factor-stimulated activation of the AC-cAMP-PKA signalling cascade (Boutillier *et al.* 1998). The ability of GLP-1 to activate the same cascade makes it a likely inducer of POMC promoter activation. Indeed, GLP-1 stimulation resulted in increased POMC promoter activity in AtT-20 cells in this study. However, the difference in magnitude between GLP-1-induced activation of the POMC and αGSU promoters may be explained, in part, by the presence of tandem CRE sites in the αGSU promoter (Jameson *et al.* 1988) thus allowing a magnified response to cAMP. The GLP-1R is known

to utilize the AC-cAMP-PKA signalling pathway (Montrose-Rafizadeh *et al.* 1999) and we were able to demonstrate downstream involvement of PKA resulting from GLP-1 stimulation using H-89 pretreatment of cells. This resulted in the partial attenuation of αGSU activation, suggesting GLP-1 mediates part of its effects via the AC-cAMP-PKA pathway, but also utilizes other signalling pathways insensitive to H-89. This observation is supported by evidence demonstrating GLP-1 activation of phosphatidylinositol 3-kinase (PI3K) in β-cells (Buteau *et al.* 1999) and the phospholipase-C-protein kinase C (PLC-PKC) pathway in COS-7 cells (Wheeler *et al.* 1993). Similar results were also obtained using the POMC promoter transfected cells pretreated with H-89 (data not shown) confirming the proposed hypothesis that the POMC promoter could respond to GLP-1-activated cAMP. Stimulation of the POMC promoter activity by GLP-1 suggests that the placement of the preproinsulin gene downstream of the promoter would result in its transcription in AtT-20 cells.

To demonstrate insulin gene transcription in this study, expression of insulin mRNA was assessed following transfection with the POMCINS-LUC plasmid in response to FSK and GLP-1. Co-transfection of the receptor with this plasmid increased the transcriptional activation of the preproinsulin gene following GLP-1 and FSK treatment. The pIRESneo plasmid was modified to express the luciferase gene downstream of the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV), which permits the translation of two open reading frames from one messenger RNA. This allows the transcription and translation of two genes, thus creating a system whereby luciferase activity in response to GLP-1 becomes an indicator of GLP-1-mediated gene insulin expression. We were able to detect insulin message in POMCINS-LUC transfected cells and reassuringly failed to do so in cells transfected with empty vector. We demonstrate, for the first time, GLP-1-induced insulin gene transcription driven by the GLP-1 responsive POMC promoter. Our results also demonstrate that the cAMP activator, FSK, is able to activate preproinsulin gene translation, concurring with earlier studies showing the action of cAMP activators and analogues in mediating insulin gene transcription in AtT-20 cells (Hughes *et al.* 1992). These data suggest that co-transfection of GLP-1R and the POMC driven preproinsulin gene may present a viable system for insulin production in response to physiological glucose concentrations.

Our model differs from previous glucose responsive AtT-20 cells expressing insulin (Hughes *et al.* 1992, Davies *et al.* 1998) in the way in which they regulate insulin expression. Previously, cells were co-transfected with the human pre-proinsulin gene and the low affinity glucose transporter isoform, GLUT2. Glucose responsivity was conferred by the glucose sensitive β-actin promoter driving the GLUT2 gene, with maximal insulin responses

only occurring in response to subphysiological concentrations of glucose. In the current study, the mechanism of responding to changes in physiological glucose levels lies not directly with a glucose responsive promoter driving insulin transcription, but indirectly with a hormone whose levels are, in turn, regulated by circulating glucose.

Using our transient expression system, we were unable to detect insulin release into the media of transfected cells. However, using an adenoviral expression system to increase expression efficiency, we were able to demonstrate GLP-1-stimulated insulin gene translation and secretion.

In summary, we have shown that AtT-20 cells are able to transcribe and express functionally active, transfected GLP-1Rs which respond to GLP-1 with resultant activation of the AC-cAMP-PKA pathway, demonstrated by increased levels of cAMP and partial inhibition of PKA activity following pretreatment with H-89. We have demonstrated GLP-1 responsiveness of the POMC promoter by means of POMC-luciferase constructs culminating in GLP-1-mediated, POMC promoter-activated proinsulin expression. Using an adenoviral expression system in AtT-20 corticotrophs, we have demonstrated GLP-1 responsiveness of the insulin promoter sufficient to result in insulin gene translation and insulin secretion. The creation of a transplantable cell line that is able to detect increased GLP-1 concentrations in response to elevated blood glucose levels and respond with a regulated production of insulin may provide an alternative approach for the treatment of diabetes.

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