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Agonist-induced internalisation of the glucagon-like peptide-1 receptor is mediated by the $G\alpha_q$ pathway

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BAPTA-AM
chlorpromazine
filipin
genistein
monodansylcadaverine (MDC)
tunicamycin
Dynasore
Go6976
PD98059
Ro318820
U73122
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ABSTRACT

The glucagon-like peptide-1 receptor (GLP-1R) is a G-protein-coupled receptor (GPCR) and an important target in the treatment of type 2 diabetes mellitus (T2DM). Upon stimulation with agonist, the GLP-1R signals through both $G\alpha_s$ and $G\alpha_q$ coupled pathways to stimulate insulin secretion. The agonist-induced GLP-1R internalisation has recently been shown to be important for insulin secretion. However, the molecular mechanisms underlying GLP-1R internalisation remain unknown. The aim of this study was to determine the role of GLP-1R downstream signalling pathways in its internalisation. Agonist-induced human GLP-1R (hGLP-1R) internalisation and activity were examined using a number of techniques including immunoblotting, ELISA, immunofluorescence and luciferase assays to determine cAMP production, intracellular Ca^{2+} accumulation and ERK phosphorylation. Agonist-induced hGLP-1R internalisation is dependent on caveolin-1 and dynamin. Inhibition of the $G\alpha_q$ pathway but not the $G\alpha_s$ pathway affected hGLP-1R internalisation. Consistent with this, hGLP-1R mutant T149M and small-molecule agonists (compound 2 and compound B), which activate only the $G\alpha_s$ pathway, failed to induce internalisation of the receptor. Chemical inhibitors of the $G\alpha_q$ pathway, PKC and ERK phosphorylation significantly reduced agonist-induced hGLP-1R internalisation. These inhibitors also suppressed agonist-induced ERK1/2 phosphorylation demonstrating that the phosphorylated ERK acts downstream of the $G\alpha_q$ pathway in the hGLP-1R internalisation. In summary, agonist-induced hGLP-1R internalisation is mediated by the $G\alpha_q$ pathway. The internalised hGLP-1R stimulates insulin secretion from pancreatic β-cells, indicating the importance of GLP-1 internalisation for insulin secretion.

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Abbreviations: 5-HT2$_A$, serotonin 5-hydroxytryptamine 2a receptor; AC, adenylyl cyclase; Ca^{2+}, calcium; cAMP, cyclic adenosine monophosphate; DABCO, 1,4-diazabicyclo[2.2.2]octane; DAG, diacylglycerol; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DMEM, Dulbecco's modified Eagle medium; DN, dominant negative; DTT, dithiothreitol; ECL, enhanced chemiluminescence; ET$_A$R, endothelin A receptor; ERK, extracellular signal-regulated kinase; FSM, full serum medium; G-CRSR, G-CSF receptor; GFP, green fluorescent protein; GLP-1, glucagon-like peptide-1; GLP-1R, GLP-1 receptor; GnRHR, gonadotropin-releasing hormone receptor; GPCR, G-protein coupled receptors; GRKs, GPCR kinases; HEK293, human embryonic kidney 293; hGLP-1R, human GLP-1R; HRP, horseradish peroxidise; IgG, immunoglobulin G; IP$_3$, inositol-1,4,5-triphosphate; IP$_3$R, inositol-1,4,5-triphosphate receptor; MAPK, mitogen-activated protein kinase; MGC, mammalian gene collection; PCR, polymerase chain reaction; PIP$_2$, phosphatidylinositol-4,5-bisphophate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PVDF, polyvinylidene fluoride; RIPA, radio-immunoprecipitation assay; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SFM, serum-free medium; T2DM, type 2 diabetes mellitus; TBS, tris buffered saline; VSVG, vesicular stomatitis virus glycoprotein..

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1. Introduction

One of the main physiological roles of glucagon-like peptide-1 (GLP-1) is to increase insulin secretion from pancreatic β-cells in a glucose-dependent manner [1,2]. This hormone is secreted by the intestinal L-cells after food intake [3]. GLP-1 exerts its physiological effects by binding to its G-protein-coupled receptor (GPCR), the GLP-1 receptor (GLP-1R). Therefore, human GLP-1R (hGLP-1R) is an important target in the treatment of type 2 diabetes mellitus (T2DM) [4].

Upon agonist binding, GPCRs undergo a conformational change and transmit extracellular signals through heterotrimeric G-proteins, which consist of Gα and Gβγ subunits [5]. The agonist occupied GLP-1R activates both $G\alpha_s$ and $G\alpha_q$ subunits [6]. The $G\alpha_s$ subunit activates adenylyl cyclase (AC), increasing cyclic adenosine monophosphate (cAMP) levels which in turn activate protein kinase A (PKA) [7]. The $G\alpha_q$ subunit activates phospholipase C (PLC), which in turn hydrolyses phosphatidylinositol-4,5-bisphophate (PIP_2) to inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to its receptor IP3 receptor (IP3R) on the endoplasmic reticulum (ER), which causes cytosolic calcium (Ca^{2+}) accumulation [8]. DAG together with intracellular Ca^{2+} activates PKC, which then induces extracellular signal-regulated kinase (ERK) phosphorylation [9,10]. ERKs are one class of mitogen-activated protein kinases (MAPKs) and their activity is regulated by phosphorylation [11]. The GLP-1R has previously been shown to activate ERK [12–15]. Further, the activation of ERK by a number of GPCRs including the M_3-muscarinic receptor has been shown to be mediated by PKC [10,16–18]. In β-cells, the increase in intracellular Ca^{2+} through the $G\alpha_q$ pathway causes secretory vesicles containing insulin to fuse to the plasma membrane and thereby increases insulin exocytosis [19]. The exocytotic insulin response caused by increased intracellular Ca^{2+} accumulation is potentiated by elevated cAMP production by coupling of agonist bound GLP-1R to the $G\alpha_s$ pathway [20].

After activation by agonist, most GPCRs internalise from the cell surface to dampen the biological response, to resensitise the desensitised receptor by recycling or to propagate signals through novel transduction pathways [21]. GPCR kinases (GRKs), arrestins and clathrin coated pits predominantly regulate agonist-induced GPCR internalisation. The agonist-activated GPCR is phosphorylated by GRKs, which facilitates the recruitment of arrestin and targets the GPCR to clathrin-coated pits for rapid internalisation [22]. However, some GPCRs such as endothelin A receptor (ET_AR) internalise in a caveolae-dependent manner [23]. The dynamin family of GTPases play an important role in agonist-induced GPCR internalisation by fission of clathrin-coated vesicles or caveolae membranes [24]. Currently, there is some confusion whether GLP-1R uses clathrin- or caveolin-mediated endocytosis for its agonist-induced internalisation. It has been reported that clathrin-coated endocytosis mediates GLP-1R internalisation and three PKC phosphorylation sites within the C-terminal domain are important for this to occur [25]. However, the GLP-1R has also shown to be internalised by caveolae-mediated endocytosis [15,26]. In agonist-stimulated pancreatic β-cells, the internalised GLP-1R colocalises with AC within endosomes and stimulates insulin secretion [27]. Therefore, a better understanding of GLP-1R internalisation is essential for introducing novel agonists of the GLP-1R in the treatment of T2DM. Although, the GLP-1R is known to activate both $G\alpha_s$ and $G\alpha_q$ coupled pathways, it is unknown as to which pathway is required for agonist induced internalisation of GLP-1R.

In this study, it was determined that agonist-induced hGLP-1R internalisation is caveolin-1- and dynamin-dependent. Furthermore, this study revealed that the $G\alpha_q$ pathway mediates agonist-induced hGLP-1R internalisation. Consistent with this, the hGLP-1R T149M mutant and small-molecule agonists (compounds 2 and B) not only failed to activate the $G\alpha_q$ pathway, but also prevented agonist-induced internalisation of the hGLP-1R. Additionally, the inhibitors of $G\alpha_q$ signalling pathway [PBP10 (a membrane-permeable PIP_2 sequestering peptide), U73122 (a PLC inhibitor), 2-aminoethoxydiphenyl borate (2-APB, an IP_3R inhibitor), BAPTA-AM (a membrane-permeable Ca^{2+} chelator)], PKC (Go6976 and Ro318820) and ERK phosphorylation (PD98059, an inhibitor for ERK phosphorylation by MAPK) reduced agonist-induced hGLP-1R internalisation. These inhibitors also suppressed ERK phosphorylation induced by hGLP-1R activation, demonstrating that the phosphorylated ERK acts downstream of the $G\alpha_q$ pathway in hGLP-1R internalisation.

2. Materials and methods

2.1. Materials

The primary antibodies used were rabbit anti-vesicular stomatitis virus glycoprotein (ab34774, VSVG) (Abcam Biochemicals, Cambridge, UK), mouse anti-green fluorescent protein (GFP) (11814460001, Roche, West Sussex, UK), mouse anti-hGLP-1R (MAB2814, R&D Systems, Abington, UK), mouse anti-CAV-1 (sc894, Santa Cruz Biotechnology, Heidelberg, Germany), rabbit anti-phospho ERK1/2 (9102, pERK1/2) and rabbit anti-ERK1/2 (9102, New England Biolabs, Hertfordshire, UK). The Cy3-conjugated anti-mouse immunoglobulin G (IgG) secondary antibody (715-165-150, Jackson Laboratories, Suffolk, UK) was used for immunofluorescence. Horseradish peroxidase (HRP)-conjugated anti-mouse (NA933) and anti-rabbit (NA934) IgG (GE Healthcare, Hertfordshire, UK) secondary antibodies were used for Western blotting. Enhanced chemiluminescence (ECL) select reagent was obtained from GE Healthcare (Hertfordshire, UK). GLP-1 (Liraglutide) was from Novo Nordisk (Sussex, UK). Compound 2 and compound B were purchased from Calbiochem (Nottingham, UK). Chemical inhibitors used were 2-APB, BAPTA-AM, chlorpromazine hydrochloride, filipin complex, genistein, monodansylcadaverine (MDC), tunicamycin (Sigma, Dorset, UK), Dynasore (Abcam Biochemicals, Cambridge, UK), Go6976, PD98059, Ro318820, U73122, U73343 (Tocris, Bristol, UK), PBP10 (Millipore, Nottingham, UK) and pentratin peptide (Thermo Scientific, Northumberland, UK). All other chemicals were from Sigma (Dorset, UK) unless otherwise stated.

2.2. Plasmids

The full-length hGLP-1RΔN23 cDNA was amplified from mammalian gene collection (MGC) clone 142053 (Source Bioscience, Nottingham, UK) by polymerase chain reaction (PCR) using high-fidelity Taq DNA polymerase (Roche Applied Science, West Sussex, UK) and sequence specific primers containing *Eco*RI restriction site and VSVG-tag coding sequence (5′ primer), and *Sal*I restriction site and no stop codon (3′ primer). SP-VSVG-hGLP-1RΔN23 cDNA was amplified by overlap PCR using VSVG-hGLP-1RΔN23 cDNA as the template, the sense primer, containing *Eco*RI restriction site, the signal peptide (SP, 1-23aa) coding sequence followed by VSVG coding sequence, and 3′ primer. The cDNA was digested with *Eco*RI and *Sal*I, and cloned in frame into the same sites of pEGFP-N1 vector (Clontech) for expression as the N-terminus VSVG-tagged (after the SP) and the C-terminus GFP-tagged fusion protein in mammalian cells. The point mutations within the hGLP-1R were generated using Quickchange II XL site-directed mutagenesis kit (Stratagene, Leicestershire, UK) and SP-VSVG-hGLP-1RΔN23-GFP plasmid as the template [28]. The dominant negative (DN) mutants of dynamin K44A, β-arrestin1 Δ319-418 and clathrin EPS15 Δ95-295 used in this study have been described previously [24,29]. The caveolae DN (CAV-1-P132L)

was obtained from Addgene (MA, USA) [30]. The $G\alpha_q$ (G188S) DN plasmid was kindly provided by Prof. Karnam S. Murthy (Virginia Commonwealth University, USA) [31]. Luciferase pGL4.29-Luc-CRE, pGL4.30-Luc-NFAT and pGL4.33-Luc-SRE reporter plasmids were from Promega (Southampton, UK).

2.3. Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells were maintained at 37 °C in a 5% CO_2 humidified environment in Dulbecco's modified Eagle medium [DMEM; serum-free medium (SFM), Biosera, East Sussex, UK] supplemented with 10% foetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin [full serum medium (FSM)]. Cells were transiently transfected for 48 h using JetPrime transfection reagent (Polyplus; 2 μl/μg DNA, VWR, Leicestershire, UK) according to the manufacturer's instructions.

2.4. ELISA

This assay is carried out as described previously with unpermeabilised cells to quantify cell surface expression of the receptor [24]. Briefly, HEK293 cells transiently transfected with plasmid DNA were serum starved for 1 h and then stimulated without or with agonist at 37 °C/5% CO_2. Where indicated, cells were incubated without or with chemical inhibitors for 30 min prior to and during stimulation with agonist at 37 °C/5% CO_2. Cells were then fixed with 4% paraformaldehyde (PFA) 5 min and non-specific binding sites blocked with 1% BSA made in TBS (1% BSA/TBS) for 45 min. Cells were incubated with the anti-hGLP-1R antibody (diluted 1:15,000) in 1% BSA/TBS for 1 h, washed with TBS and then incubated with HRP-conjugated anti-mouse IgG (diluted 1:5000) in 1% BSA/TBS for 1 h. Cells were washed and developed using 1-step Ultra TMB-ELISA substrate (Bio-Rad, Herts, UK) for 15 min and the reaction stopped by adding an equal volume of 2 M sulphuric acid. The optical density was read at 450 nm using a plate reader.

2.5. Immunofluorescence

Intracellular localisation of hGLP-1R expression was assessed by immunofluorescence as described previously [24]. Briefly, cells were serum starved for 1 h and where indicated cells were pre-incubated without or with inhibitors at the indicated concentration for 30 min. Cells were then incubated with the anti-hGLP-1R mouse monoclonal antibody (diluted 1:5000) in 1% BSA/SFM for 1 h at 4 °C and then stimulated without or with agonist in the presence of inhibitor at 37 °C/5% CO_2. Cells were then fixed with 4% PFA for 30 min. Cells were permeabilised with 0.2% Triton X-100 made in PBS for 10 min, blocked in blocking buffer [1% BSA made in wash buffer (0.1% Triton X-100 in PBS)] for 30 min and then incubated with Cy3-conjugated anti-mouse antibody (diluted 1:200 in blocking buffer) for 1 h. Cells were then washed 3 times with wash buffer and incubated with DAPI (D8417, 4′,6-diamidino-2-phenylindole dihydrochloride, 1 mg/ml) diluted 1:2000 in PBS to stain nucleus. Coverslips were mounted on glass microscopic slides using mounting solution (0.1 M Tris–HCl, pH 8.5, 10% Mowiol 50% glycerol) containing 2.5% DABCO (1,4-diazabicyclo (2.2.2) octane). Immunofluorescence staining was visualised using a Zeiss LSM710 confocal microscope fitted with a 63× oil immersion lens [28].

2.6. cAMP assay

Cells were serum starved for 1 h and then stimulated without or with 100 nM GLP-1 for 1 h at 37 °C/5% CO_2 in the presence of 0.25 mM phosphodiesterase inhibitor Ro201724. Cells were lysed and cAMP levels in the cell lysates were assessed using the cAMP direct immunoassay kit (Abcam, Cambridge, UK).

2.7. cAMP, Ca^{2+} and ERK luciferase assay

HEK293 cells cotransfected with hGLP-1R plasmid and luciferase reporter plasmid for cAMP (pGL4.29-Luc-CRE) or intracellular Ca^{2+} (pGL4.30-Luc-NFAT) or ERK phosphorylation (pGL4.33-Luc-SRE) were treated with increasing concentrations of agonist for 4 h (cAMP and ERK) or 8 h (Ca^{2+}) at 37 °C/5% CO_2. After incubation, an equal volume of ONE-Glo™ lysis buffer containing luciferase substrate (Promega, Southampton, UK) was added to each well and luminescence measured using a plate reader in accordance with the manufacturer's instructions.

2.8. Cell lysates

To make cell lysates, HEK293 cells expressing hGLP-1R were washed 3 times with ice-cold PBS and lysed in ice-cold modified RIPA lysis buffer (10 mM Tris–HCl, pH 7.5, containing 10 mM EDTA, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate and 150 mM NaCl) with 1% mammalian protease inhibitors. Cell lysates were incubated at 4 °C for 15 min and then centrifuged at 22,000 × g for 10 min at 4 °C. The supernatant was collected and ½ volume of 3× SDS–polyacrylamide gel electrophoresis (PAGE) sample loading buffer [75 mM Tris–HCl, pH 6.8, containing 3% SDS, 30% glycerol, 0.003% bromophenol blue and 0.3 M dithiothreitol (DTT)] was added and left at room temperature for 1 h. These cell lysates used to detect hGLP-1R expression by immunoblotting using the anti-GFP and anti-VSVG antibodies.

For assessing ERK phosphorylation, HEK293 cells expressing hGLP-1R were lysed in ice-cold modified RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, containing 0.2 M NaCl, 10 mM $MgCl_2$, 0.1% SDS, 0.5% sodium deoxycholate, 1% TritonX-100 and 5% glycerol) with 1% mammalian protease inhibitors. Cell lysates were incubated at 4 °C for 15 min and centrifuged at 22,000 × g for 10 min at 4 °C. The supernatant was collected and ¼ volume of 5× SDS–PAGE sample loading buffer (125 mM Tris–HCl, pH 6.8, containing 5% SDS, 50% glycerol, 0.005% bromophenol blue and 5% β-mercaptoethanol) was added and heated at 100 °C for 5 min. These cell lysates were used to detect phosphorylated ERK and total ERK by immunoblotting using the anti-pERK1/2 and anti-ERK1/2 antibodies.

2.9. Immunoblotting

Proteins were separated in an SDS–PAGE gel by electrophoresis and transferred onto polyvinylidene fluoride (PDVF) membrane. Membranes were blocked with TBS-T (TBS with 0.1% Tween-20) containing 5% milk powder (blocking buffer) for 1 h and immunoblotted with the anti-GFP mouse antibody (diluted 1:500 in blocking buffer) to assess protein expression levels or the anti-pERK1/2 rabbit antibody (diluted 1:1000 in blocking buffer) to assess ERK1/2 phosphorylation for 1 h at room temperature or overnight at 4 °C. Membranes were washed and then incubated with the HRP-conjugated anti-mouse or anti-rabbit secondary antibody (diluted 1:2500 in blocking buffer) for 1 h at room temperature. Membranes were then incubated in ECL select substrate and bands visualised using the ChemiDoc™ XRS system (Bio-Rad). Blots probed with the anti-GFP mouse antibody were stripped with Western blot stripping buffer (Thermo Scientific) and reprobed with the anti-VSVG rabbit antibody (diluted 1:1000) in blocking buffer to assess protein expression levels. Blots probed with the anti-pERK1/2 rabbit antibody were stripped and reprobed with the anti-ERK1/2 rabbit antibody (diluted 1:1000 in blocking buffer) to assess ERK1/2 phosphorylation [32].

2.10. Data analysis

Data were analysed using the GraphPad Prism program. All data are presented as means ± SEM of three independent experiments. Statistical comparisons between a control and test value was made by a two-tailed unpaired student's *t*-test. Statistical analysis between multiple groups was determined by the Bonferroni's post hoc test after one-way or two-way ANOVA. A *p*-value <0.05 was considered statistically significant. Concentration response curves were also fitted using Prism, according to a standard logistic equation. Confocal images shown in the figures are representative of 190–200 transfected cells from three different experiments. Similarly, immunoblotting data shown in the figures are representative of three independent experiments.

3. Results

3.1. HGLP-1R is internalised by caveolae-mediated endocytosis

The concentration (Fig. 1) and time dependency (Fig. 2) of agonist (GLP-1)-induced internalisation of hGLP-1R was assessed by ELISA (A) and immunofluorescence (B) using HEK293 cells transiently transfected with the receptor. The addition of 100 nM GLP-1 to cells led to time-dependent internalisation of the hGLP-1R with maximum internalisation observed 60 min after addition of agonist (Fig. 2A). When added to the cells for 60 min, GLP-1 induced hGLP-1R internalisation with an EC_{50} of 33.65 nM and a maximum 76% internalisation being observed at 1 μM GLP-1 (Fig. 1A). Immunofluorescence staining of cells confirmed the concentration (Fig. 1B) and time dependence (Fig. 2B) of agonist-induced internalisation of hGLP-1R, where a reduction in cell surface expression of the receptor and an increase in intracellular endosomal-like vesicles was observed in cells stimulated with agonist. Furthermore, the kinetics of agonist-induced internalisation of the hGLP-1R with the N-terminal VSVG tag (after the SP) and the C-terminal GFP tag (SP-VSVG-hGLP-1RΔN23-GFP) is identical to that of the untagged or tagged with either the N-terminal VSVG or the C-terminal GFP hGLP-1R (data not shown). Unless otherwise indicated, agonist treatment of 100 nM GLP-1 for 60 min was used in further experiments.

Next, the role of clathrin, caveolin and dynamin in agonist-induced internalisation of the hGLP-1R was analysed. Most GPCRs internalise in either a clathrin- or caveolae-dependent manner [23,33]. Dynamin regulates both clathrin- and caveolae-mediated endocytosis through fission of the endocytosed vesicles [34]. To determine whether agonist-induced hGLP-1R internalisation is mediated by clathrin or caveolae and dynamin, HEK293 cells expressing the hGLP-1R were either cotransfected with DN mutants (Fig. 3A) or treated with chemical inhibitors (Fig. 3B) of clathrin, caveolae or dynamin and stimulated with agonist and analysed by ELISA and immunofluorescence. GLP-1R internalisation in the presence of chemical inhibitors or DN mutants is shown as percentage of that in the absence of the treatment in brackets next to the treatment.

The DN mutant of dynamin (dynamin K44A), which affects both clathrin- and caveolae-mediated endocytosis, significantly reduced (33.69 ± 3.84%, $p < 0.001$) agonist-induced hGLP-1R internalisation (Fig. 3A). However, clathrin DN mutants, β-arrestin1 Δ319-418 (89.80 ± 6.83%, $p > 0.05$) and EPS15 Δ95-295 (86.30 ± 5.05%, $p > 0.05$), had little effect on the receptors internalisation. In contrast, the DN mutant of caveolin-1 (Cav-1-P132L) abolished hGLP-1R internalisation (0.01 ± 0.16%, $p < 0.001$). Immunofluorescence analysis confirmed the inhibition of hGLP-1R internalisation by dynamin and cavelin-1 DN mutants (Fig. 3A). Inhibitors of clathrin-mediated endocytosis, chlorpromazine (95.50 ± 2.77%, $p > 0.05$) and MDC (94.68 ± 3.39%, $p > 0.05$), had no significant effect on hGLP-1R internalisation. However, chemical inhibitors of dynamin, dynasore (31.1 ± 4.92%, $p < 0.001$), and caveolae-mediated endocytosis, genistein (22.76 ± 1.83%, $p < 0.001$), and filipin (0.1 ± 0.09%, $p < 0.001$) inhibited agonist-induced hGLP-1R internalisation (Fig. 3B). These observations were supported by immunofluorescence analysis where a reduction in agonist-induced intracellular accumulation of hGLP-1R in endosomes was observed in cells treated with caveolae inhibitors. These results together demonstrate that agonist induced hGLP-1R internalisation is caveolae- and dynamin-dependent.

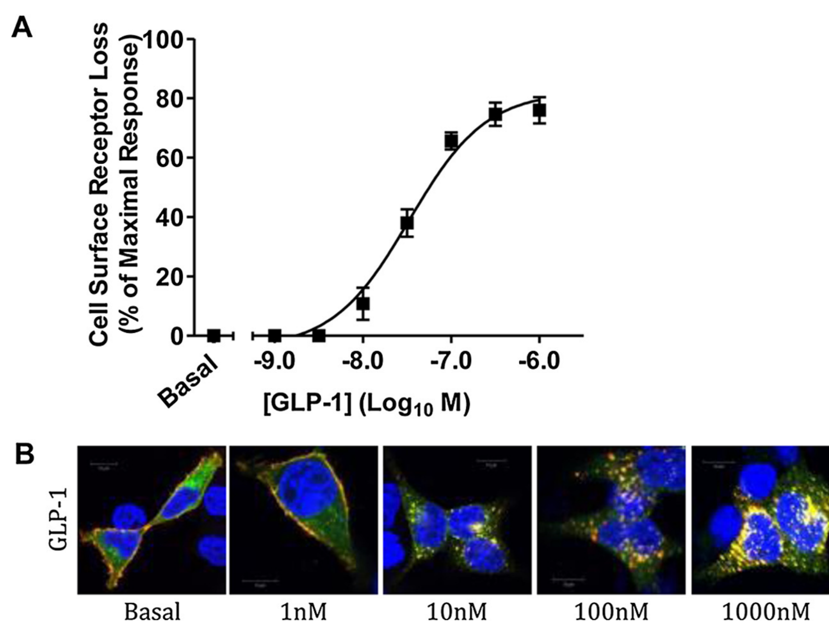

Fig. 1. Concentration-dependent stimulation of hGLP-1R internalisation by GLP-1. HEK293 cells expressing the hGLP-1R were stimulated with GLP-1 at the indicated concentration for 60 min and hGLP-1R internalisation was assessed by ELISA (A) and immunofluorescence (B) using the anti-hGLP-1R antibody. In immunofluorescence, EGFP (green) and the anti-hGLP-1R antibody (red) overlay is shown in yellow and nuclear staining with DAPI in blue. Data are mean ± SEM, *n* = 3.

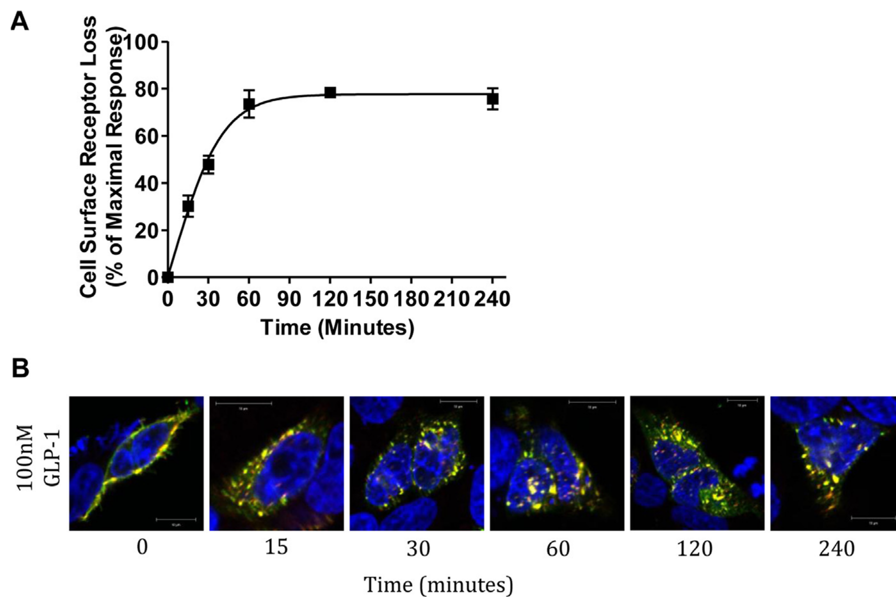

Fig. 2. Time-dependent stimulation of hGLP-1R internalisation by GLP-1. HGLP-1R internalisation stimulated with 100 nM GLP-1 for the indicated time was assessed by ELISA (A) and immunofluorescence (B) using the anti-hGLP-1R antibody. In immunofluorescence, EGFP (green) and the anti-hGLP-1R antibody (red) overlay is shown in yellow and nuclear staining with DAPI in blue. Data are mean + SEM, $n = 3$.

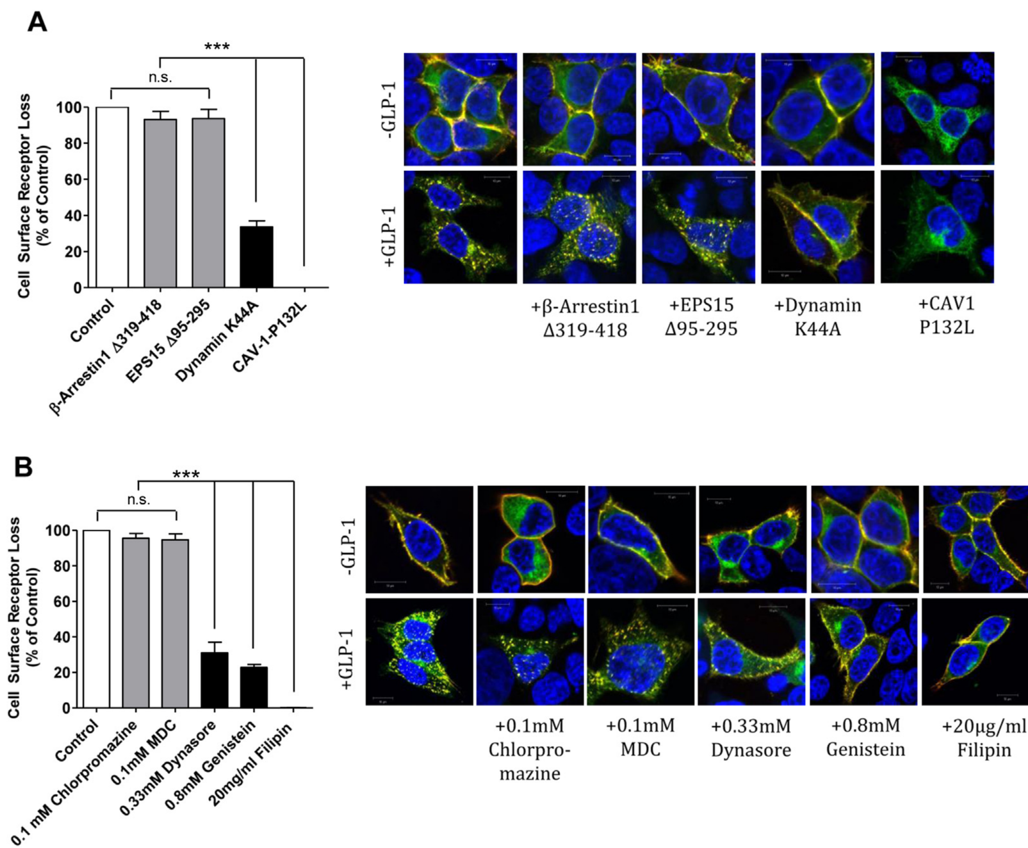

Fig. 3. HGLP-1R is internalised by caveolae-mediated endocytosis. HEK293 cells expressing the hGLP-1R were either cotransfected with dominant negative mutants (A) or treated with chemical inhibitors (B) as indicated and agonist-induced internalisation quantified by ELISA (left panel) and immunofluorescence (right panel) using anti-hGLP-1R antibody. In immunofluorescence, EGFP (green) and anti-hGLP-1R antibody (red) overlay is shown in yellow and nuclear staining with DAPI in blue. Data are mean ± SEM, $n = 3$, n.s. $p > 0.05$; $^{***}p < 0.001$.

3.2. Agonist-induced hGLP-1R internalisation is dependent on the $G\alpha_q$ pathway

Upon agonist binding, the hGLP-1R acts through the $G\alpha_s$ coupled pathway to stimulate cAMP production and the $G\alpha_q$ coupled pathway to increase intracellular Ca^{2+} levels [6]. However, the involvement of these two pathways in agonist-induced hGLP-1R internalisation is unknown. Therefore, whether agonist-induced internalisation of the hGLP-1R was dependent on the $G\alpha_s$ or $G\alpha_q$ pathway was determined using a number of activators and inhibitors of both the pathways (Fig. 4A). The $G\alpha_s$ pathway activator forskolin ($99.59 \pm 0.98\%$, $p > 0.05$) and inhibitors, SQ22536 ($98.43 \pm 2.9\%$, $p > 0.05$) and H89 ($104.29 \pm 5.61\%$, $p > 0.05$), had no effect on hGLP-1R agonist-induced internalisation when used at the concentration that is optimal to induce (10 μM forskolin) or inhibit (0.1 mM SQ22536) cAMP production or inhibit PKA activation (10 mM H89) (Fig. 4B). In contrast, the $G\alpha_q$ (G188S) DN mutant inhibited agonist-induced hGLP-1R internalisation ($34 \pm 2.93\%$, $p < 0.001$). This was further confirmed by immunofluorescence (Fig. 4C). These results suggest hGLP-1R internalisation may require the $G\alpha_q$ pathway.

The requirement of the $G\alpha_q$ pathway for agonist-induced hGLP-1R internalisation was further assessed by using hGLP-1R T149M mutant [35] and small-molecule agonists (compounds 2 and B) of the hGLP-1R that are known to activate only the $G\alpha_s$ pathway [36–40]. The T149M mutants total protein expression (determined by immunoblotting; Fig. 5A]), cell surface expression [assessed by ELISA (Fig. 5B; $106.9 \pm 4.25\%$, $p > 0.05$) and immunofluorescence (Fig. 5G)]; the receptor activity [assessed by cAMP response (Fig. 5C; $107.5 \pm 0.36\%$, $p > 0.05$)] were similar to that of the hGLP-1R wild-type. However, agonist-induced hGLP-1R internalisation was abolished by the T149M mutation [assessed by ELISA (Fig. 5F; $1.47 \pm 0.81\%$, $p < 0.001$) and immunofluorescence (Fig. 5G)]. Moreover, intracellular Ca^{2+} accumulation (Fig. 5D; $23.12 \pm 1.46\%$, $p < 0.001$) and ERK phosphorylation (Fig. 5E; $4.14 \pm 1.5\%$, $p < 0.001$) in agonist-stimulated cells expressing the hGLP-1R T149M mutant were significantly reduced. Taken together, these results suggest that the T149M mutation of hGLP-1R affects agonist-induced internalisation of the receptor and the activation of $G\alpha_q$ coupled pathway, indicating the importance of the $G\alpha_q$ pathway for agonist-induced hGLP-1R internalisation.

The small-molecule agonists, compounds 2 and B, were also used to assess their effects on agonist-induced cAMP production (Fig. 6A), intracellular Ca^{2+} accumulation (Fig. 6B), ERK phosphorylation (Fig. 6C) and hGLP-1R internalisation [by ELISA (Fig. 6D) and immunofluorescence (Fig. 6E)]. No hGLP-1R internalisation was observed in cells stimulated with compound 2 ($0.33 \pm 0.17\%$, $p < 0.001$) or compound B ($0.08 \pm 0.04\%$,

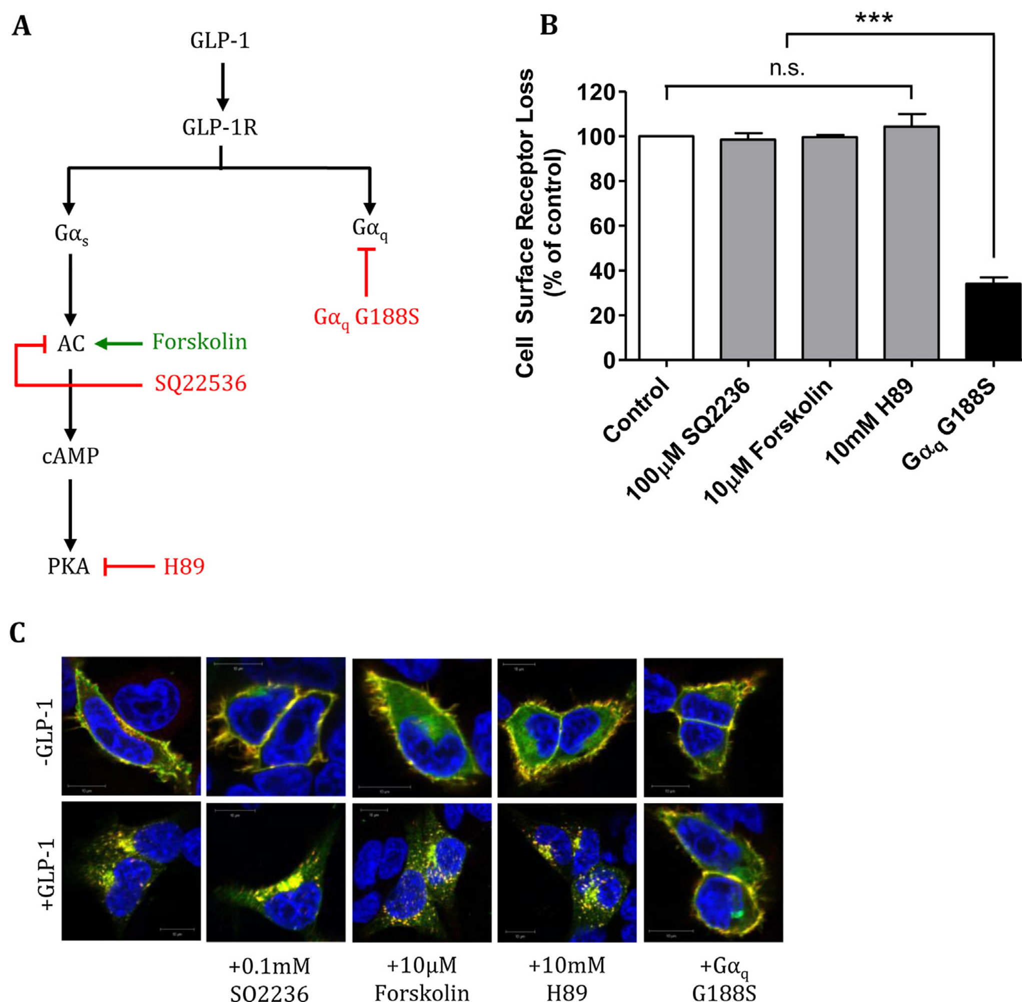

Fig. 4. HGLP-1R internalisation is dependent on the $G\alpha_q$ pathway. (A) Simplified scheme showing various inhibitors or activators of the $G\alpha_s$-coupled pathway or $G\alpha_q$ used to assess their effect on hGLP-1R internalisation. HGLP-1R internalisation in HEK293 cells treated with the inhibitors as indicated was quantified using anti-hGLP-1R antibody by ELISA (B) and immunofluorescence (C). In immunofluorescence, EGFP (green) and anti-hGLP-1R antibody (red) overlay is shown in yellow and nuclear staining with DAPI in blue. Data are mean ± SEM, $n = 3$, n.s. $p > 0.05$; $^{***}p < 0.001$.

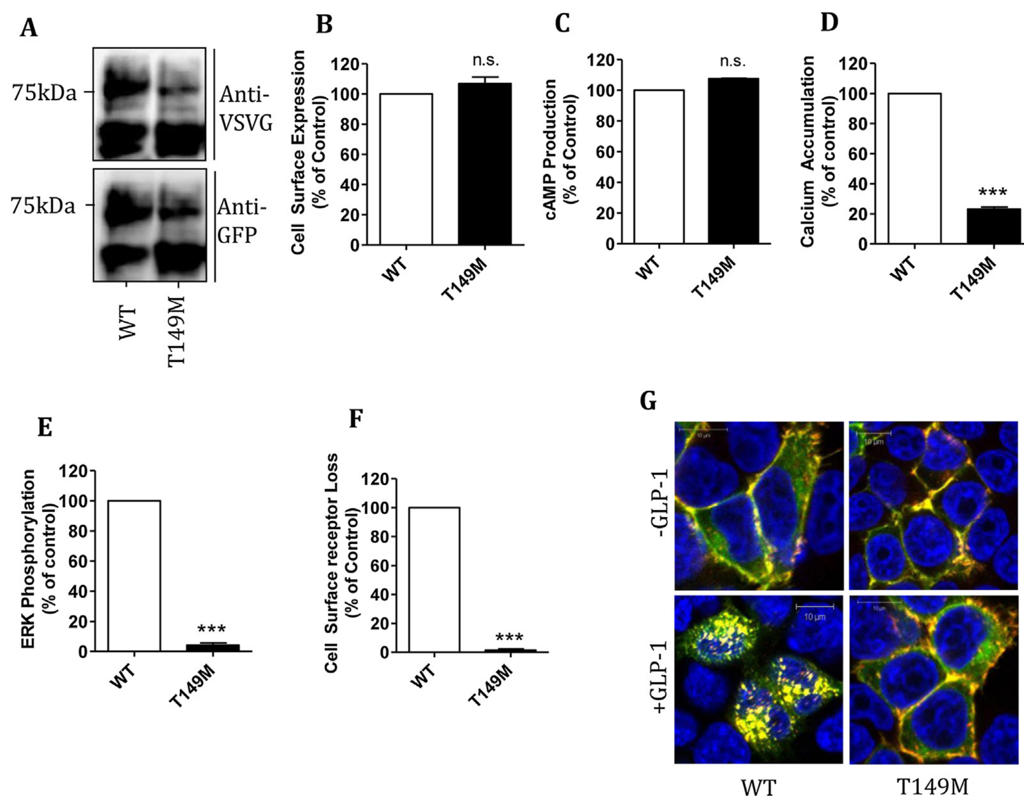

Fig. 5. The T149M mutation inhibits agonist-induced hGLP-1R internalisation. HEK293 cells were transfected with hGLP-1R wild-type (WT) or T149M for 2 days. (A) Total protein expression of WT or T149M was assessed by immunoblotting using anti-GFP and anti-VSVG antibodies. (B) HGLP-1R WT or T149M cell surface expression was quantified by ELISA using anti-hGLP-1R antibody. HEK293 cells expressing hGLP-1R WT or T149M were stimulated with agonist and measured cAMP accumulation (C), intracellular Ca^{2+} accumulation (D) and ERK phosphorylation (E) to assess hGLP-1R activity. HGLP-1R WT or T149M internalisation in HEK293 cells was assessed by ELISA (F) and immunofluorescence (G) using anti-hGLP-1R antibody. In immunofluorescence, EGFP (green) and anti-hGLP-1R antibody (red) overlay is shown in yellow and nuclear staining with DAPI in blue. Data are mean ± SEM, $n = 3$, n.s. $p > 0.05$; $^{***}p < 0.001$.

$p < 0.001$). Immunofluorescence supported these observations by demonstrating the reduction in hGLP-1R internalisation in cells treated with small-molecule agonists, compounds 2 and B. As observed previously by other studies [36–40], both small-molecule agonists have induced cAMP production but not intracellular Ca^{2+} accumulation or ERK phosphorylation. Stimulation with optimal concentration of compound 2 resulted in only 7.27 ± 7.27% ($p < 0.001$) intracellular Ca^{2+} accumulation and 20.01 ± 8.52% ($p < 0.001$) ERK phosphorylation when compared to that of GLP-1 stimulation. Compound B caused only 16.77 ± 8.96% ($p < 0.001$) intracellular Ca^{2+} accumulation and 13.63 ± 7.76% ($p < 0.001$) ERK phosphorylation. These results show that small-molecule agonists, compounds 2 and B, are unable to internalise hGLP-1R or induce intracellular Ca^{2+} accumulation and ERK phosphorylation, suggesting that the $G\alpha_q$ pathway and ERK phosphorylation may be important for agonist-induced hGLP-1R internalisation.

3.3. Inhibition of the $G\alpha_q$ pathway prevents agonist-induced hGLP-1R internalisation

The $G\alpha_q$ pathway causes intracellular Ca^{2+} accumulation by activating PLC, which hydrolyses PIP_2 to IP_3 and DAG. IP_3 binds to the IP_3R on the ER and increases cytosolic Ca^{2+} levels. An increase in intracellular Ca^{2+} levels leads to PKC activation, which then regulates many signalling pathways including ERK phosphorylation [8,9,11]. To study the importance of the $G\alpha_q$ pathway in agonist-stimulated hGLP-1R internalisation, the concentration-dependent effect of PIP_2 sequestering membrane-permeable peptide (PBP10), PLC inhibitor (U73122), an IP_3R inhibitor (2-APB) and a membrane-permeable chelator for intracellular calcium (BAPTA-AM) on agonist-induced hGLP-1R internalisation was analysed (Fig. 7A–D). PBP10 inhibited internalisation of the receptor in a concentration-dependent manner and maximal inhibition was observed in the presence of 30 μM PBP10 (8.1 ± 2.59%, $p < 0.001$). U73122 treatment also resulted in a concentration-dependent inhibition of hGLP-1R internalisation with maximal inhibition at 100 μM (1.93 ± 1.84%, $p < 0.001$). 2-APB and BAPTA-AM also inhibited agonist-induced hGLP-1R internalisation in a concentration-dependent manner and resulted in maximal inhibition at 4 mM (4.81 ± 1.21%, $p < 0.001$) and 1 mM (8.86 ± 2.43%, $p < 0.001$), respectively. These observations were confirmed by immunofluorescence where inhibition of agonist-induced internalisation of hGLP-1R was evident. The negative controls, penetratin for PBP10 (membrane-permeable peptide that does not bind PIP_2), U73343 for U73122 (inactive isomer of U73122) and BAPTA-AM + Ca^{2+} for BAPTA-AM (BAPTA-AM saturated with Ca^{2+} is inactive in chelating intracellular Ca^{2+}) showed no effect on agonist-induced hGLP-1R internalisation (data not shown). Taken together, these results demonstrate that the $G\alpha_q$ pathway regulates agonist-induced hGLP-1R internalisation.

3.4. Effect of the inhibition of PKC and ERK phosphorylation on hGLP-1R internalisation

The accumulation of intracellular Ca^{2+} in the $G\alpha_q$ pathway activates PKC, which has previously been shown to link the receptors activation to ERK phosphorylation [10,16–18,41–45]. Therefore, the effect of PKC inhibitors, Go6976 and Ro318220, and ERK inhibitor, PD98059, on agonist-induced internalisation of hGLP-1R was analysed (Fig. 8A–C). The PKC

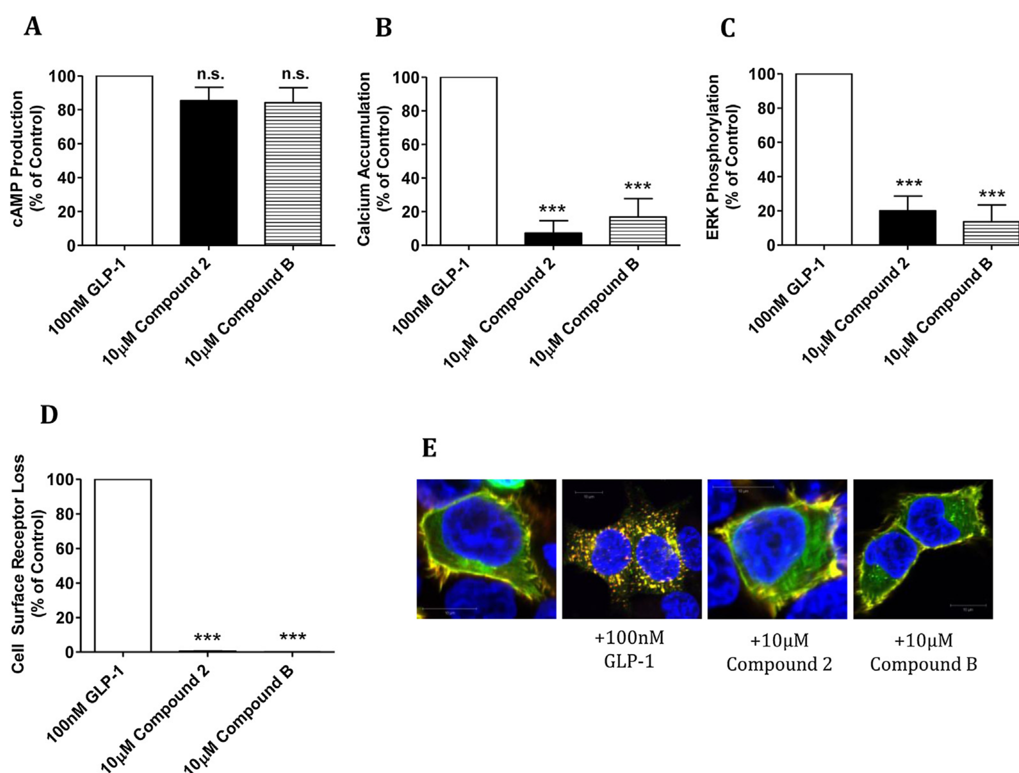

Fig. 6. Small-molecule agonists activate the $G\alpha_s$ pathway and inhibit hGLP-1R internalisation. HEK293 cells cotransfected with plasmids for hGLP-1R and the luciferase reporter for cAMP (pGL4.29-Luc-CRE), intracellular Ca^{2+} (pGL4.30-Luc-NFAT) or ERK phosphorylation (pGL4.33-Luc-SRE) were stimulated with GLP-1, compound 2 and compound B to assess cAMP generation (A), intracellular Ca^{2+} accumulation (B), ERK phosphorylation (C) and the receptor internalisation by ELISA (D) and immunofluorescence (E). In immunofluorescence, EGFP (green) and anti-hGLP-1R antibody (red) overlay is shown in yellow and nuclear staining with DAPI in blue. Data normalised to percentage stimulation of GLP-1 and are shown as mean ± SEM, $n = 3$, n.s. $p > 0.05$; $^{***}p < 0.001$.

inhibitors, Go6976 and Ro318220, inhibited agonist-induced hGLP-1R internalisation in a concentration-dependent manner, with maximal inhibition at 100 μM (46.02 ± 4.33%, $p < 0.001$ and 24.07 ± 5.41%, $p < 0.001$, respectively). The ERK inhibitor PD98059 also inhibited hGLP-1R internalisation in a concentration-dependent manner, with maximal inhibition at 100 μM (9.28 ± 2.92%, $p < 0.001$). These observations were confirmed by immunofluorescence where inhibition of agonist-induced internalisation of hGLP-1R by these chemicals was evident. This data suggest that the agonist-induced internalisation of the hGLP-1R is dependent on both the activation of PKC and ERK phosphorylation.

3.5. Effect of the $G\alpha_q$ pathway inhibitors on GLP-1-induced ERK phosphorylation and cAMP production

Since the activation of calcium-dependent PKC and ERK is required for agonist-induced hGLP-1R internalisation and the activation of $G\alpha_q$ pathway leads to an increase in intracellular Ca^{2+} levels, we determined whether the $G\alpha_q$ pathway regulates internalisation of the receptor through ERK phosphorylation. For this purpose, the effect of inhibitors of the $G\alpha_q$ pathway on agonist-induced ERK phosphorylation was assessed (Fig. 9A). The negative controls, penetratin (for PBP10), U73343 (for U73122) and BAPTA-AM + Ca^{2+} (for BAPTA-AM) showed no effect on ERK phosphorylation (107.9 ± 3.55, 99.63 ± 9.93, 96 ± 12.71%, $p > 0.05$, respectively). In contrast, the PIP_2 sequester, PBP10, reduced ERK phosphorylation to 36.8 ± 8.22% ($p < 0.001$). U73122, the inhibitor of PLC, almost abolished ERK phosphorylation to 4.78 ± 0.69% ($p < 0.001$). The IP_3R inhibitor, 2-APB, was also significantly inhibited ERK phosphorylation (46.59 ± 8.02%, $p < 0.001$). Only 22.3 ± 8.84% ($p < 0.001$) ERK phosphorylation was seen in the presence of BAPTA-AM, the chelator of intracellular Ca^{2+}. The PKC inhibitors, Go6976 and Ro318820, almost abolished agonist-induced ERK phosphorylation to 9.34 ± 1.8% ($p < 0.001$) and 14.90 ± 2.16% ($p < 0.001$) respectively. Lastly, the MAPK inhibitor, PD98059, inhibited ERK phosphorylation (16.03 ± 8.1%, $p < 0.001$), as expected. Since the $G\alpha_s$ pathway mediates cAMP generation, the $G\alpha_q$ pathway specific inhibitors had no effect ($p > 0.05$) on agonist-induced cAMP production (Fig. 9B). Taking these results together with the effect of the inhibitors of $G\alpha_q$ pathway, PKC activation and ERK phosphorylation on hGLP-1R internalisation further indicate that the $G\alpha_q$ pathway regulates agonist-induced hGLP-1R internalisation via ERK phosphorylation.

4. Discussion

Upon activation by agonist binding, many GPCRs are internalised to reduce the activity of the receptor. The internalised GPCRs are subjected to one of two sorting fates. They are either recycled back to the plasma membrane resulting in resensitisation of receptors or transported to lysosomes and proteolysed leading to long-term attenuation of signalling (down-regulation) [46]. Agonist-induced GLP-1R internalisation and recycling has been shown in transfected fibroblasts and in insulinomas [47]. Currently, it is unknown by which pathway GLP-1R internalisation occurs and how cells respond to drugs after the initial internalisation phase. With the possibility of drugs being produced which are administered once a week or once a month rather than once daily. The effects these drugs have on GLP-1R cell surface expression, internalisation, recycling and degradation needs to be understood for the half-life of these compounds to be prolonged further and for the effect of 'long-acting-release' drugs to be successful [48]. In pancreatic β-cells, an increase in cytosolic Ca^{2+} causes the release of insulin by exocytosis [19]. The increase in intracellular Ca^{2+}-mediated insulin secretion is potentiated by elevated cAMP levels

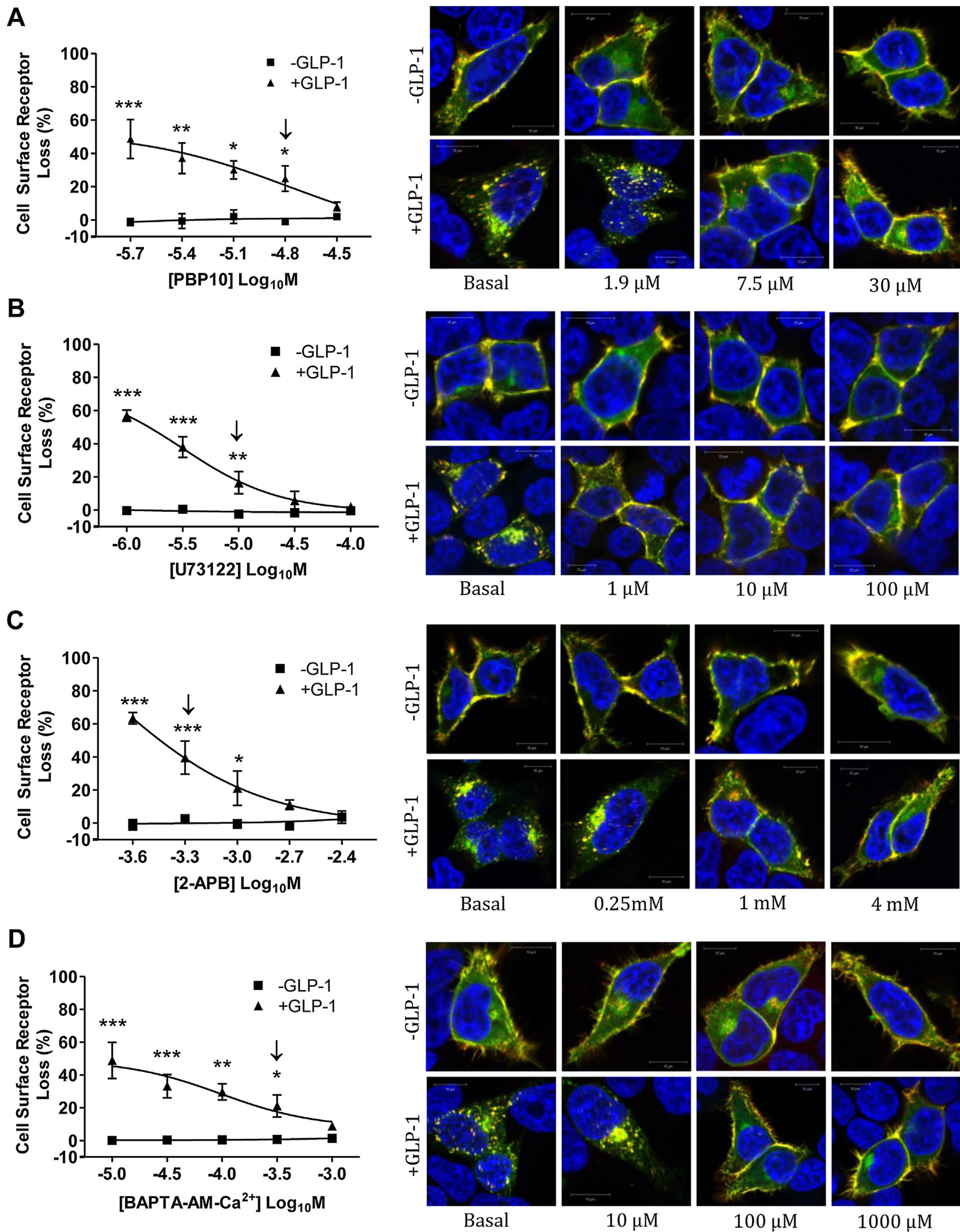

Fig. 7. Effect of the $G\alpha_q$ pathway inhibitors on agonist-induced hGLP-1R internalisation. Agonist-induced hGLP-1R internalisation in HEK293 cells treated with various concentrations of chemical inhibitors PBP10 (A), U73122 (B), 2-APB (C) and BAPTA-AM (D) was assessed by ELISA (left panel) and immunofluorescence (right panel) using anti-hGLP-1R antibody. In immunofluorescence, EGFP (green) and anti-hGLP-1R antibody (red) overlay is shown in yellow and nuclear staining with DAPI in blue. Data are mean ± SEM, $n = 3$, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (↓ denotes the concentration used in Fig. 9).

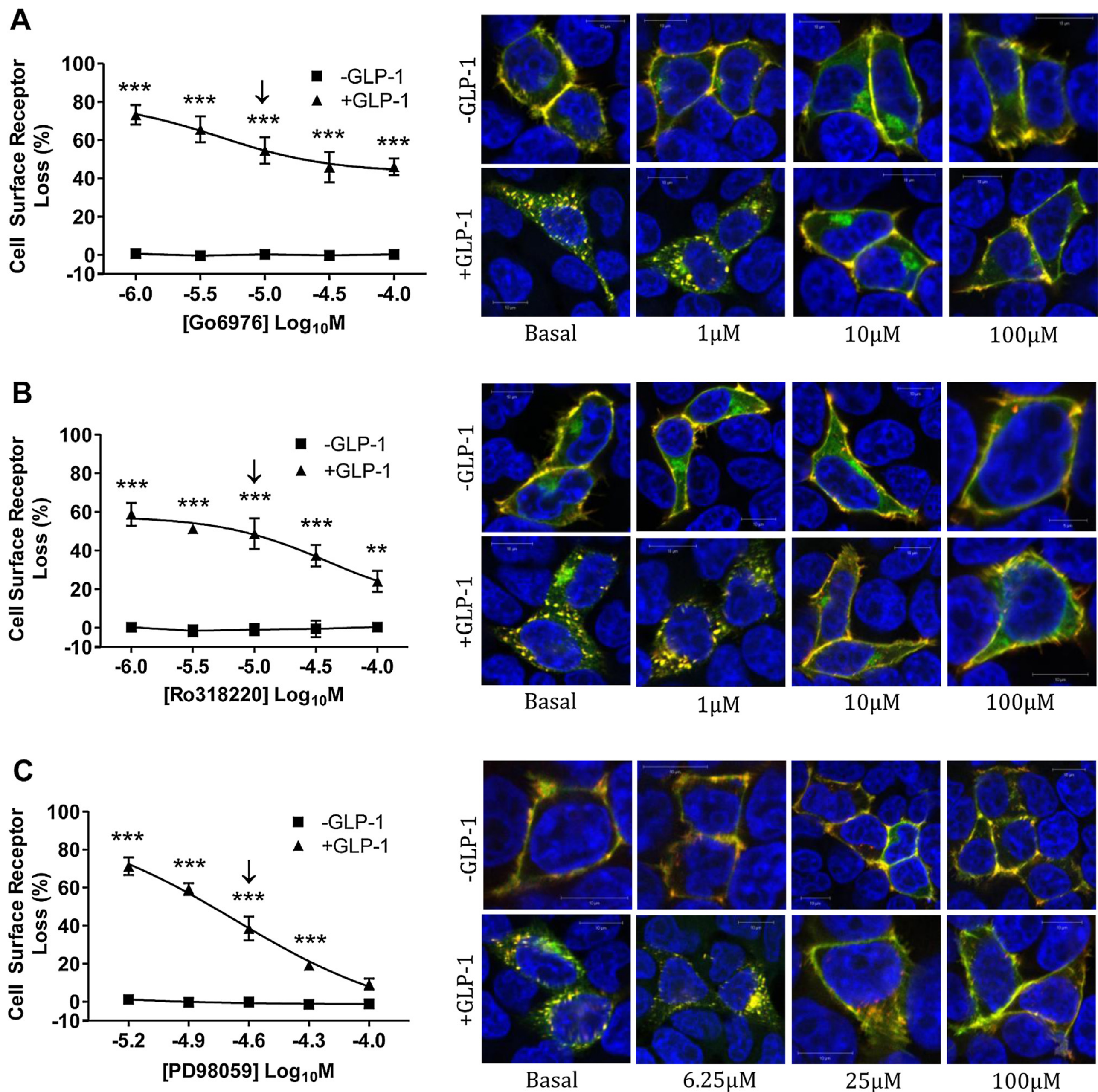

Fig. 8. Effect of PKC and ERK phosphorylation inhibitors on agonist-induced hGLP-1R internalisation. Agonist-induced internalisation of HGLP-1R in HEK293 cells treated with various concentrations of PKC inhibitor Go6976 (A) or Ro318220 (B), or ERK phosphorylation inhibitor PD98059 (C) was quantified by ELISA (left panel) and immunofluorescence (right panel) using anti-hGLP-1R antibody. In immunofluorescence, EGFP (green) and anti-hGLP-1R antibody (red) overlay is shown in yellow and nuclear staining with DAPI in blue. Data are mean ± SEM, $n = 3$, $^{**}p < 0.01$; $^{***}p < 0.001$ (↓ denotes the concentration used in Fig. 9).

[20]. Upon agonist stimulation, the internalised GLP-1R has been shown to colocalise with AC on endosomes and stimulate insulin secretion from pancreatic β-cells demonstrating the importance of hGLP-1R internalisation for insulin secretion [27]. Therefore, agonist-induced internalisation of the hGLP-1R into intracellular compartments of the cell is important for regulation of the receptors activity [24,49]. This study systematically analysed the involvement of the $G\alpha_q$ pathway in agonist-induced GLP-1R internalisation in HEK293 model cell line.

In this study, the hGLP-1R was demonstrated, by various approaches, to internalise by caveolae-mediated endocytosis. This is consistent with previous findings where caveolin-1 has been shown to interact with the hGLP-1R and other GPCRs for targeting, internalisation and recycling of the receptor [15,23]. The GLP-1R has been shown to activate both the $G\alpha_s$-coupled pathway to generate cAMP and the $G\alpha_q$-coupled pathway to cause accumulation of intracellular Ca^{2+} [6]. In this study, inhibition of the $G\alpha_q$, but not $G\alpha_s$, signalling pathway markedly reduced agonist-induced hGLP-1R internalisation, indicating a critical role for the $G\alpha_q$ pathway in hGLP-1R internalisation. The serotonin 5-hydroxytryptamine 2a (5-HT2$_A$) receptor and the gonadotropin-releasing hormone receptor (GnRHR) also couple to and internalise through the $G\alpha_q$ pathway [50–53]. The T149M mutation in the hGLP-1R, which originally identified in a T2DM patient with impaired insulin secretion [54], has been shown to reduce agonist responsiveness [35]. In this study, HEK293 cells expressing either hGLP-1R or its mutant T149M demonstrated similar cAMP generation when stimulated with GLP-1, indicating the mutation had no effect on

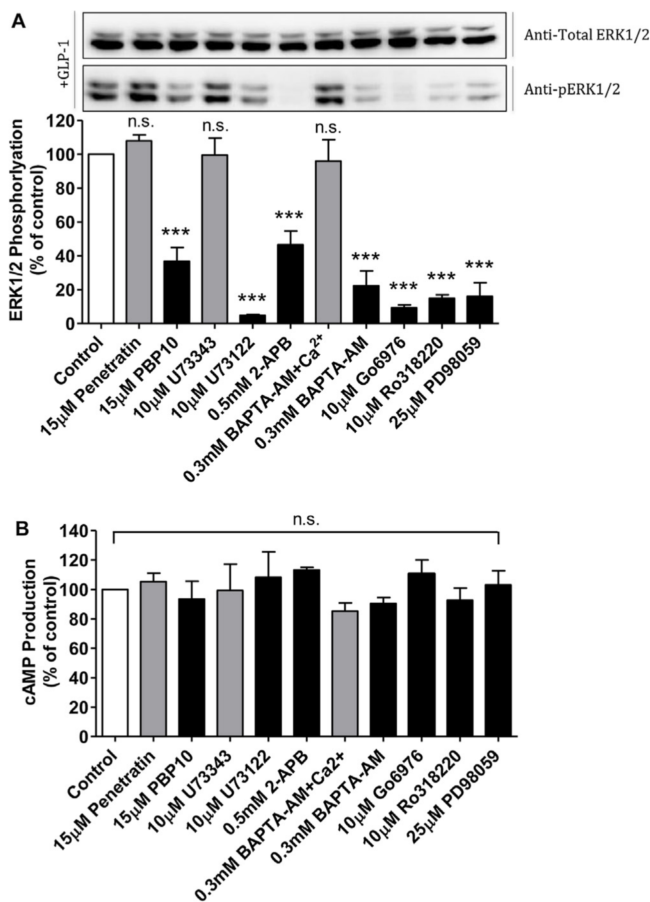

Fig. 9. Effect of the $G\alpha_q$ pathway inhibition on agonist stimulated ERK phosphorylation and cAMP production. HEK293 cells expressing hGLP-1R were stimulated with agonist in the presence of $G\alpha_q$ pathway inhibitors and assessed ERK1/2 phosphorylation (A) and cAMP production (B). ERK1/2 phosphorylation measured by immunoblotting (upper panel) and quantified ERK1/2 phosphorylation by densitometry and normalising to total ERK1/2 levels (lower panel). Data are mean ± SEM, $n = 3$, n.s. $p > 0.05$; $^{***}p < 0.001$.

either agonist binding to the receptor or its activity. This study also demonstrates that the mutation, instead, significantly reduces agonist-induced GLP-1R internalisation by affecting intracellular Ca^{2+} accumulation and ERK phosphorylation and strongly suggests this as a possible cause for the patient's reduced insulin secretion found in the T2DM patient with T149M mutation [54]. Like the T149M mutant, small-molecule agonists (compounds 2 and B) neither activated the $G\alpha_q$ pathway nor induced hGLP-1R internalisation. This is consistent with previous studies that demonstrated compounds 2 and B activate only the $G\alpha_s$ pathway [36–40]. Both compounds 2 and B differ from the orthosteric agonist (GLP-1) in inducing GLP-1R internalisation and downstream signalling, implying 'biased agonism' for both small-molecule agonists [55,56]. Further, cAMP produced in response to hGLP-1R stimulation is important for glucose-stimulated insulin secretion [57]. It has recently been shown that pharmacological inhibition of GLP-1R internalisation attenuates agonist-mediated insulin secretion [27]. This is because the internalised GLP-1R associates with AC on endosomes to generate cAMP required for insulin secretion. It is therefore a possibility that the T149M mutation and small-molecule agonists (compounds 2 and B) affect insulin secretion by inhibiting GLP-1R internalisation and thereby endosomal cAMP generation.

In this study, chemical inhibition of PLC activation and intracellular Ca^{2+} accumulation affected agonist-induced internalisation of the hGLP-1R, further demonstrating the GLP-1R couples and internalises through the $G\alpha_q$ pathway [8]. Since the increase in intracellular Ca^{2+} levels downstream of agonist-stimulated GLP-1R activates PKC [8], the effect of two PKC inhibitors, Go6976 and Ro318220, on the agonist-induced internalisation of the GLP-1R was determined. The PKC family consists of several isoforms in humans that are activated in either a Ca^{2+}-dependent or independent manner. The inhibitor Go6976 is selective for Ca^{2+}-dependent PKC isoforms [58], whereas Ro318220 is a broad-spectrum PKC inhibitor, which inhibits both Ca^{2+}-dependent and Ca^{2+}-independent PKC isoforms [59]. The inhibition of agonist-induced GLP-1R by both the PKC inhibitors demonstrate the importance of Ca^{2+}-dependent PKC isoforms for the receptors internalisation. It is also important to note that the GLP-1R contains three PKC phosphorylation sites within the C-terminal domain which are important for internalisation [25]. Removal of these phosphorylation sites has been shown to prevent agonist-induced GLP-1R internalisation demonstrating the importance of PKC phosphorylation of the receptor in GLP-1R internalisation. The δ-opioid receptor also requires the activation of PKC to allow phosphorylation of the receptor for internalisation [60]. In this study, the inhibition of PKC prevented not only agonist-induced internalisation, but also ERK phosphorylation, indicating that PKC may play a role in GLP-1R internalisation by phosphorylating the receptor as well as regulating the phosphorylation of ERK.

The ERK is phosphorylated by receptor tyrosine kinases in Src- and Ras-dependent manners [9,10,61–63]. However, GPCRs phosphorylate ERK through the $G\alpha_s$, $G\alpha_i$ or $G\alpha_q$ pathways depending on the receptor type and environment [64]. The ERK phosphorylation that occurs through the $G\alpha_q$ pathway is highly dependent on both intracellular Ca^{2+} accumulation and PKC activation [10]. Inhibition of PKC in the α_{1B} adrenergic receptor [65] abolished the receptor-mediated ERK phosphorylation, demonstrating PKC acts upstream of ERK. The results obtained in this study strongly suggest GLP-1-mediated ERK phosphorylation occurs downstream of the PKC activation. This suggests that the accumulation of intracellular Ca^{2+} and thereby activation of

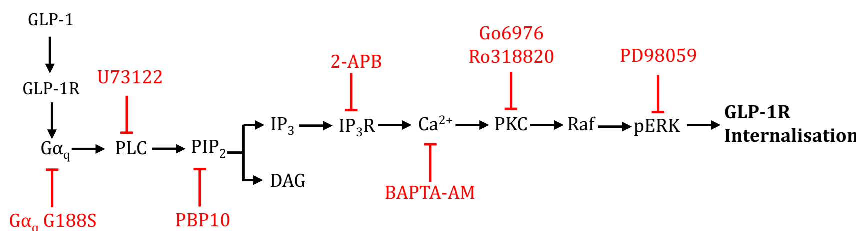

Fig. 10. Schematic representation of the pathway of agonist-induced hGLP-1R internalisation deduced from this study.

PKC is able to induce ERK phosphorylation, linking activation of the receptor to ERK phosphorylation. ERK phosphorylation has also been shown to play an important role in the internalisation of GPCRs such as the δ-opioid receptor, G-CSFR and 5-$HT2_A$ receptor [50,66–69]. Although how phosphorylated ERK regulates hGLP-1R internalisation is unclear, it is possible that ERK phosphorylation may regulate internalisation of the receptor through interacting with and/or phosphorylating various signalling molecules involved in agonist-induced GPCR internalisation. Consistent with this, we have recently shown that the phosphorylated ERK1/2 regulates preadipocyte migration through dynamin [70]. It is therefore possible that dynamin may also function downstream of ERK in hGLP-1R internalisation.

In conclusion, these results demonstrate that caveolin-1 plays an important role in hGLP-1R trafficking to the cell surface and its internalisation in HEK293 cells. Upon agonist activation, the hGLP-1R signals through the $G\alpha_q$ pathway to hydrolyse PIP_2 by PLC to generate IP_3. IP_3 binds the IP_3R and increases cytosolic Ca^{2+} accumulation, which then leads to the activation of PKC. In turn, this leads to the phosphorylation of ERK via the MAPK pathway [8]. In this study, the inhibitors of the $G\alpha_q$ pathway, PKC activation and ERK phosphorylation affected not only hGLP-1R internalisation but also ERK phosphorylation, indicating that together they play a vital role in agonist-induced internalisation of the receptor (Fig. 10). In this study, the hGLP-1R T149M mutation, which was previously found in Japanese patient with T2DM and impaired insulin secretion, and small-molecule agonists (compounds 2 and B), of GLP-1R also inhibited agonist-induced hGLP-1R internalisation. This suggests an important role for hGLP-1R internalisation in insulin secretion.

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References

[1] Doyle ME, Egan JM. Mechanisms of action of glucagon-like peptide 1 in the pancreas. Pharmacology and Therapeutics 2007;113:546–93.
[2] Holz GG, Leech CA, Heller RS, Castonguay M, Habener JF. cAMP-dependent mobilization of intracellular Ca stores by activation of ryanodine receptors in pancreatic β-cells. The Journal of Biological Chemistry 1999;274:14147–56.
[3] Thompson A, Kanamarlapudi V. Type 2 diabetes mellitus and glucagon like peptide-1 receptor signalling. Clinical and Experimental Pharmacology 2013;3. DOI: 2161-1459.1000138.
[4] Gallwitz B. The evolving place of incretin-based therapies in type 2 diabetes. Pediatric Nephrology 2010;25:1207–17.
[5] Cabrera-Vera TM, Vanhauwe J, Thomas TO, Medkova M, Preininger A, Mazzoni MR, et al. Insights into G protein structure, function, and regulation. Endocrine Reviews 2003;24:765–81.
[6] Montrose-Rafizadeh C, Avdonin P, Garant MJ, Rodgers BD, Kole S, Yang H, et al. Pancreatic glucagon-like peptide-1 receptor couples to multiple G proteins and activates mitogen-activated protein kinase pathways in Chinese hamster ovary cells. Endocrinology 1999;140:1132–40.
[7] Bos JL. Epac: a new cAMP target and new avenues in cAMP research. Nature Reviews Molecular Cell Biology 2003;4:733–8.
[8] Werry TD, Wilkinson GF, Willars GB. Mechanisms of cross-talk between G-protein-coupled receptors resulting in enhanced release of intracellular Ca^{2+}. Biochemical Journal 2003;374:281–96.
[9] Hawes BE, van Biesen T, Koch WJ, Luttrell LM, Lefkowitz RJ. Distinct pathways of Gi- and Gq-mediated mitogen-activated protein kinase activation. The Journal of Biological Chemistry 1995;270:17148–53.
[10] Budd DC, Willars GB, McDonald JE, Tobin AB. Phosphorylation of the G(q/11)-coupled M-3-muscarinic receptor is involved in receptor activation of the ERK-1/2 mitogen-activated protein kinase pathway. Journal of Biological Chemistry 2001;276:4581–7.
[11] Cobb MH, Goldsmith EJ. How MAP kinases are regulated. The Journal of Biological Chemistry 1995;270:14843–46.
[12] Jolivalt CG, Fineman M, Deacon CF, Carr RD, Calcutt NA. GLP-1 signals via ERK in peripheral nerve and prevents nerve dysfunction in diabetic mice. Diabetes Obesity and Metabolism 2011;13:990–1000.
[13] Quoyer J, Longuet C, Broca C, Linck N, Costes S, Varin E, et al. GLP-1 mediates antiapoptotic effect by phosphorylating Bad through a beta-arrestin 1-mediated ERK1/2 activation in pancreatic beta-cells. The Journal of Biological Chemistry 2010;285:1989–2002.
[14] Koole C, Wootten D, Simms J, Valant C, Sridhar R, Woodman OL, et al. Allosteric ligands of the glucagon-like peptide 1 receptor (GLP-1R) differentially modulate endogenous and exogenous peptide responses in a pathway-selective manner: implications for drug screening. Molecular Pharmacology 2010;78:456–65.
[15] Syme CA, Zhang L, Bisello A. Caveolin-1 regulates cellular trafficking and function of the glucagon-like Peptide 1 receptor. Molecular Endocrinology 2006;20:3400–11.
[16] Budd DC, Rae A, Tobin AB. Activation of the mitogen-activated protein kinase pathway by a Gq/11-coupled muscarinic receptor is independent of receptor internalization. The Journal of Biological Chemistry 1999;274: 12355–60.
[17] Wylie PG, Challiss RA, Blank JL. Regulation of extracellular-signal regulated kinase and c-Jun N-terminal kinase by G-protein-linked muscarinic acetylcholine receptors. The Biochemical Journal 1999;338(Pt 3):619–28.
[18] Kim JY, Yang MS, Oh CD, Kim KT, Ha MJ, Kang SS, et al. Signalling pathway leading to an activation of mitogen-activated protein kinase by stimulating M3 muscarinic receptor. The Biochemical Journal 1999;337(Pt 2):275–80.
[19] Holz GG. Epac A new cAMP-binding protein in support of glucagon-like peptide-1 receptor-mediated signal transduction in the pancreatic beta-cell. Diabetes 2004;53:5–13.
[20] Holst JJ, Gromada J. Role of incretin hormones in the regulation of insulin secretion in diabetic and nondiabetic humans. American Journal of Physiology Endocrinology and Metabolism 2004;287:E199–206.
[21] Hanyaloglu AC, von Zastrow M. Regulation of GPCRs by endocytic membrane trafficking and its potential implications. Annual Review of Pharmacology and Toxicology 2008;48:537–68.
[22] Gurevich VV, Gurevich EV. The structural basis of arrestin-mediated regulation of G-protein-coupled receptors. Pharmacology and Therapeutics 2006;110:465–502.
[23] Chini B, Parenti M. G-protein coupled receptors in lipid rafts and caveolae: how, when and why do they go there. Journal of Molecular Endocrinology 2004;32:325–38.
[24] Kanamarlapudi V, Thompson A, Kelly E, Lopez Bernal A. ARF6 activated by the LHCG receptor through the cytohesin family of guanine nucleotide exchange factors mediates the receptor internalization and signaling. The Journal of Biological Chemistry 2012;287:20443–55.
[25] Widmann C. Internalization and homologous desensitization of the GLP-1 receptor depend on phosphorylation of the receptor carboxyl tail at the same three sites. Molecular Endocrinology 1997;11:1094–102.
[26] Williams TM, Lisanti MP. The caveolin proteins. Genome Biology 2004;5:214.
[27] Kuna RS, Girada SB, Asalla S, Vallentyne J, Maddika S, Patterson JT, et al. Glucagon-like peptide-1 receptor-mediated endosomal cAMP generation promotes glucose-stimulated insulin secretion in pancreatic beta-cells. American Journal of Physiology Endocrinology and Metabolism 2013;305:E161–70.
[28] Kanamarlapudi V. EFA6R requires C-terminal targeting to the plasma membrane to promote cytoskeletal rearrangement through the activation of ARF6. The Journal of Biological Chemistry 2014. http://dx.doi.org/10.1074/jbc.M113.534156.
[29] Mundell SJ, Matharu AL, Pula G, Roberts PJ, Kelly E. Agonist-induced internalization of the metabotropic glutamate receptor 1a is arrestin- and dynamin-dependent. Journal of Neurochemistry 2001;78:546–51.
[30] Holst JJ, Vilsboll T, Deacon CF. The incretin system and its role in type 2 diabetes mellitus. Molecular and Cellular Endocrinology 2009;297:127–36.
[31] Huang J, Zhou H, Mahavadi S, Sriwai W, Murthy KS. Inhibition of Galphaq-dependent PLC-beta1 activity by PKG and PKA is mediated by phosphorylation of RGS4 and GRK2. American Journal of physiology Cell Physiology 2007;292:C200–8.
[32] Davies JC, Bain SC, Kanamarlapudi V. ADP-ribosylation factor 6 regulates endothelin-1-induced lipolysis in adipocytes. Biochemical Pharmacology 2014;90:406–13.
[33] Luttrell LM, Lefkowitz RJ. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. Journal of Cell Science 2002;115:455–65.
[34] Le PU, Nabi IR. Distinct caveolae-mediated endocytic pathways target the Golgi apparatus and the endoplasmic reticulum. Journal of Cell Science 2003;116:1059–71.
[35] Beinborn M, Worrall CI, McBride EW, Kopin AS. A human glucagon-like peptide-1 receptor polymorphism results in reduced agonist responsiveness. Regulatory Peptides 2005;130:1–6.
[36] Sloop KW, Willard FS, Brenner MB, Ficorilli J, Valasek K, Showalter AD, et al. Novel small molecule glucagon-like peptide-1 receptor agonist stimulates insulin secretion in rodents and from human islets. Diabetes 2010;59: 3099–107.
[37] Coopman K, Huang Y, Johnston N, Bradley SJ, Wilkinson GF, Willars GB. Comparative effects of the endogenous agonist glucagon-like peptide-1 (GLP-1)-(7-36) amide and the small-molecule ago-allosteric agent compound 2 at the GLP-1 receptor. The Journal of Pharmacology and Experimental Therapeutics 2010;334:795–808.

[38] Irwin N, Flatt PR, Patterson S, Green BD. Insulin-releasing and metabolic effects of small molecule GLP-1 receptor agonist 6,7-dichloro-2-methylsulfonyl-3-*N-tert*-butylaminoquinoxaline. European Journal of Pharmacology 2010;628: 268–73.

[39] Knudsen LB, Kiel D, Teng M, Behrens C, Bhumralkar D, Kodra JT, et al. Small-molecule agonists for the glucagon-like peptide 1 receptor. Proceedings of the National Academy of Sciences of the United States of America 2007;104:937–42.

[40] Wootten D, Savage EE, Willard FS, Bueno AB, Sloop KW, Christopoulos A, et al. Differential activation and modulation of the glucagon-like peptide-1 receptor by small molecule ligands. Molecular Pharmacology 2013;83:822–34.

[41] Watanabe T, Waga I, Honda Z, Kurokawa K, Shimizu T. Prostaglandin F2 alpha stimulates formation of p21ras-GTP complex and mitogen-activated protein kinase in NIH-3T3 cells via Gq-protein-coupled pathway. The Journal of Biological Chemistry 1995;270:8984–90.

[42] Zou Y, Komuro I, Yamazaki T, Aikawa R, Kudoh S, Shiojima I, et al. Protein kinase C, but not tyrosine kinases or Ras, plays a critical role in angiotensin II-induced activation of Raf-1 kinase and extracellular signal-regulated protein kinases in cardiac myocytes. The Journal of Biological Chemistry 1996;271:33592–97.

[43] Venkatakrishnan G, Salgia R, Groopman JE. Chemokine receptors CXCR-1/2 activate mitogen-activated protein kinase via the epidermal growth factor receptor in ovarian cancer cells. The Journal of Biological Chemistry 2000;275:6868–75.

[44] Soltoff SP, Avraham H, Avraham S, Cantley LC. Activation of P2Y2 receptors by UTP and ATP stimulates mitogen-activated kinase activity through a pathway that involves related adhesion focal tyrosine kinase and protein kinase C. The Journal of Biological Chemistry 1998;273:2653–60.

[45] Tapia JA, Ferris HA, Jensen RT, Garcia LJ. Cholecystokinin activates PYK2/CAKbeta by a phospholipase C-dependent mechanism and its association with the mitogen-activated protein kinase signaling pathway in pancreatic acinar cells. The Journal of Biological Chemistry 1999;274:31261–71.

[46] Marchese A, Chen C, Kim YM, Benovic JL. The ins and outs of G protein-coupled receptor trafficking. Trends in Biochemical Sciences 2003;28:369–76.

[47] Widmann C, Dolci W, Thorens B. Agonist-induced internalization and recycling of the glucagon-like peptide-1 receptor in transfected fibroblasts and in insulinomas. Biochemical Journal 1995;310:203–14.

[48] Gedulin BR, Smith P, Prickett KS, Tryon M, Barnhill S, Reynolds J, et al. Dose-response for glycaemic and metabolic changes 28 days after single injection of long-acting release exenatide in diabetic fatty Zucker rats. Diabetologia 2005;48:1380–5.

[49] Bhaskaran RS, Ascoli M. The post-endocytotic fate of the gonadotropin receptors is an important determinant of the desensitization of gonadotropin responses. Journal of Molecular Endocrinology 2005;34:447–57.

[50] Bhattacharyya S, Puri S, Miledi R, Panicker MM. Internalization and recycling of 5-HT2A receptors activated by serotonin and protein kinase C-mediated mechanisms. Proceedings of the National Academy of Sciences of the United States of America 2002;99:14470–75.

[51] Kramer HK, Poblete JC, Azmitia EC. Activation of protein kinase C (PKC) by 3,4-methylenedioxymethamphetamine (MDMA) occurs through the stimulation of serotonin receptors and transporter. Neuropsychopharmacology 1997;17:117–29.

[52] Nash MS, Wood JPM, Osborne NN. Protein kinase C activation by serotonin potentiates agonist-induced stimulation of cAMP production in cultured rat retinal pigment epithelial cells. Experimental Eye Research 1997;64:249–55.

[53] McArdle CA, Franklin J, Green L, Hislop JN. The gonadotrophin-releasing hormone receptor: signalling, cycling and desensitisation. Archives of Physiology and Biochemistry 2002;110:113–22.

[54] Tokuyama Y, Matsui K, Egashira T, Nozaki O, Ishizuka T, Kanatsuka A. Five missense mutations in glucagon-like peptide 1 receptor gene in Japanese population. Diabetes Research and Clinical Practice 2004;66:63–9.

[55] Rominger DH, Cowan CL, Gowen-MacDonald W, Violin JD. Biased ligands: pathway validation for novel GPCR therapeutics. Current Opinion in Pharmacology 2014;16:108–15.

[56] Violin JD, Crombie AL, Soergel DG, Lark MW. Biased ligands at G-protein-coupled receptors: promise and progress. Trends in Pharmacological Sciences 2014;35:308–16.

[57] Lee YS, Jun HS. Anti-diabetic actions of glucagon-like peptide-1 on pancreatic beta-cells. Metabolism: Clinical and Experimental 2014;63:9–19.

[58] Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, et al. Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. The Journal of Biological Chemistry 1993;268:9194–7.

[59] Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. The Biochemical Journal 2000;351:95–105.

[60] Xiang B, Yu GH, Guo J, Chen L, Hu W, Pei G, et al. Heterologous activation of protein kinase C stimulates phosphorylation of delta-opioid receptor at serine 344, resulting in beta-arrestin- and clathrin-mediated receptor internalization. The Journal of Biological Chemistry 2001;276:4709–16.

[61] Lopez-Ilasaca M, Crespo P, Pellici PG, Gutkind JS, Wetzker R. Linkage of G protein-coupled receptors to the MAPK signaling pathway through PI 3-kinase gamma. Science 1997;275:394–7.

[62] Luttrell LM, Hawes BE, van Biesen T, Luttrell DK, Lansing TJ, Lefkowitz RJ. Role of c-Src tyrosine kinase in G protein-coupled receptor- and Gbetagamma subunit-mediated activation of mitogen-activated protein kinases. The Journal of Biological Chemistry 1996;271:19443–50.

[63] Crespo P, Xu N, Simonds WF, Gutkind JS. Ras-dependent activation of MAP kinase pathway mediated by G-protein beta gamma subunits. Nature 1994;369:418–20.

[64] Gutkind JS. The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades. The Journal of Biological Chemistry 1998;273:1839–42.

[65] Della Rocca GJ, van Biesen T, Daaka Y, Luttrell DK, Luttrell LM, Lefkowitz RJ. Ras-dependent mitogen-activated protein kinase activation by G protein-coupled receptors. Convergence of Gi- and Gq-mediated pathways on calcium/calmodulin, Pyk2, and Src kinase. The Journal of Biological Chemistry 1997;272:19125–32.

[66] Eisinger DA, Schulz R. Extracellular signal-regulated kinase/mitogen-activated protein kinases block internalization of delta-opioid receptors. The Journal of Pharmacology and Experimental Therapeutics 2004;309:776–85.

[67] Daaka Y, Luttrell LM, Ahn S, Della Rocca GJ, Ferguson SS, Caron MG, et al. Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. The Journal of Biological Chemistry 1998;273:685–8.

[68] Ward AC, van Aesch YM, Schelen AM, Touw IP. Defective internalization and sustained activation of truncated granulocyte colony-stimulating factor receptor found in severe congenital neutropenia/acute myeloid leukemia. Blood 1999;93:447–58.

[69] Hunter MG, Avalos BR. Deletion of a critical internalization domain in the G-CSFR in acute myelogenous leukemia preceded by severe congenital neutropenia. Blood 1999;93:440–6.

[70] Davies CB, Tamaddon-Jahromi S, Jannoo R, Venkateswarlu K. Cytohesin 2/ARF6 regulates preadipocyte migration through the activation of ERK1/2. Biochemical Pharmacology 2014. http://dx.doi.org/10.1016/j.bcp.2014.09.023.