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RESEARCH PAPER

β_2-Adrenoceptors increase translocation of GLUT4 via GPCR kinase sites in the receptor C-terminal tail

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BACKGROUND AND PURPOSE

β-Adrenoceptor stimulation induces glucose uptake in several insulin-sensitive tissues by poorly understood mechanisms.

EXPERIMENTAL APPROACH

We used a model system in CHO-K1 cells expressing the human β_2-adrenoceptor and glucose transporter 4 (GLUT4) to investigate the signalling mechanisms involved.

KEY RESULTS

In CHO-K1 cells, there was no response to β-adrenoceptor agonists. The introduction of β_2-adrenoceptors and GLUT4 into these cells caused increased glucose uptake in response to β-adrenoceptor agonists. GLUT4 translocation occurred in response to insulin and β_2-adrenoceptor stimulation, although the key insulin signalling intermediate PKB was not phosphorylated in response to β_2-adrenoceptor stimulation. Truncation of the C-terminus of the β_2-adrenoceptor at position 349 to remove known phosphorylation sites for GPCR kinases (GRKs) or at position 344 to remove an additional PKA site together with the GRK phosphorylation sites did not significantly affect cAMP accumulation but decreased β_2-adrenoceptor-stimulated glucose uptake. Furthermore, inhibition of GRK by transfection of the βARKct construct inhibited β_2-adrenoceptor-mediated glucose uptake and GLUT4 translocation, and overexpression of a kinase-dead GRK2 mutant (GRK2 K220R) also inhibited GLUT4 translocation. Introducing β_2-adrenoceptors lacking phosphorylation sites for GRK or PKA demonstrated that the GRK sites, but not the PKA sites, were necessary for GLUT4 translocation.

CONCLUSIONS AND IMPLICATIONS

Glucose uptake in response to activation of β_2-adrenoceptors involves translocation of GLUT4 in this model system. The mechanism is dependent on the C-terminus of the β_2-adrenoceptor, requires GRK phosphorylation sites, and involves a signalling pathway distinct from that stimulated by insulin.

Abbreviations

β_2-adrenoceptor (–)GRK, β_2-adrenoceptor lacking GRK phosphorylation sites; β_2-adrenoceptor(–)PKA, β_2-adrenoceptor lacking both PKA phosphorylation sites.; CHO-K1, Chinese hamster ovary K1 cells; GLUT, glucose transporter; GRK, G protein-coupled receptor kinase; PI3K, phosphatidylinositol-3 kinase; t344-β_2-adrenoceptor, β_2-adrenoceptor truncated at amino acid 344; t349-β_2-adrenoceptor, β_2-adrenoceptor truncated at amino acid 349; wt, wild-type

Introduction

The endogenous catecholamines adrenaline and noradrenaline regulate whole body glucose and glycogen metabolism by activation of different adrenoceptor subtypes in the heart, skeletal muscle, adipocytes, brain, pancreas and liver. Adrenoceptor agonists can stimulate glucose uptake in several tissues independently of insulin, both *in vivo* (Abe *et al.*, 1993; Liu

and Stock, 1995) and *in vitro* (Nevzorova *et al.*, 2002; Chernogubova *et al.*, 2004; 2005; Hutchinson *et al.*, 2005; 2007; Hutchinson and Bengtsson, 2006; Nevzorova *et al.*, 2006; Kanda and Watanabe, 2007; Ngala *et al.*, 2008). We have previously shown that both α_1- and β_2-adrenoceptors increase glucose uptake in L6 skeletal muscle cells, but the major contribution occurs from activation of β_2-adrenoceptors (Nevzorova *et al.*, 2002; 2006; Hutchinson *et al.*, 2005). Our studies indicated that the insulin and β_2-adrenoceptor pathways may not be additive and utilize similar mechanisms to increase glucose uptake. Unlike insulin, however, stimulation of either α_1- or β_2-adrenoceptors in skeletal muscle cells failed to activate the key insulin-signalling intermediate PKB (Hutchinson *et al.*, 2005; Nevzorova *et al.*, 2006).

β_2-Adrenoceptors classically couple to the stimulatory Gαs protein, which activates adenylyl cyclase to increase cAMP levels and activate PKA. This response is rapidly desensitized by receptor phosphorylation [β-adrenoceptors are phosphorylated at serine or threonine residues within consensus sites in the third intracellular loop and C-terminal tail by PKA and GPCR kinases (GRKs)], interaction with scaffolding proteins such as arrestins, and receptor internalization (Benovic *et al.*, 1985; 1986; Hausdorff *et al.*, 1989; Hardin and Lima, 1999; Moffett *et al.*, 2001). A recent study of endogenous β_2-adrenoceptors in HEK293 cells shows that phosphorylation of the β_2-adrenoceptor by PKA plays little or no role in desensitization of cAMP responses (Violin *et al.*, 2008). Instead, desensitization is mediated by GRK phosphorylation of the β_2-adrenoceptor, combined with PKA activation of PDE4 leading to degradation of cAMP. Phosphorylated receptors bind arrestin-2 and/or arrestin-3, and accumulate in clathrin-coated pits for endocytosis or recycling to the plasma membrane (Laporte *et al.*, 1999, Hanyaloglu and von Zastrow, 2008). Importantly, it has been established that phosphorylation by GRKs and binding of arrestins plays an important role in directing agonist-activated β_2-adrenoceptor signalling towards novel G protein-independent pathways such as activation of MAP kinases (Lefkowitz and Shenoy, 2005; Shenoy *et al.*, 2006; DeWire *et al.*, 2007; Barki-Harrington and Rockman, 2008; Kendall and Luttrell, 2009).

Of the seven GRK isoforms characterized to date, GRK-2, 3, 5, and 6 are widely distributed, and can be divided functionally into two distinct classes (Pitcher *et al.*, 1998). GRK2 and GRK3 are recruited to the cell membrane only following receptor activation and in the presence of G$\beta\gamma$ subunits (Pitcher *et al.*, 1995). In contrast, GRK5 and GRK6 are constitutively associated with the cell membrane: the N-terminal domain of GRK5 binds to phosphatidylinositol-bisphosphate (PIP_2), and basic residues within the C-terminus bind non-specifically to phospholipids. GRK6 is tethered to the cell membrane by palmitoylation of three cysteine residues in the C-terminus (Stoffel *et al.*, 1994). In this study, we investigated the role of GRKs on β_2-adrenoceptor-mediated glucose uptake and glucose transporter 4 (GLUT4) translocation using CHO-K1 cells.

CHO-K1 cells have been used previously to study glucose uptake and GLUT4 translocation mediated by Gαq-coupled receptors (Kishi *et al.*, 1996) and insulin receptors (Blero *et al.*, 2001). CHO-K1 cells express very low (or no) endogenous levels of β-adrenoceptors, in contrast to HEK293 cells. Glucose transport in CHO-K1 cells is normally controlled by GLUT1, with no endogenous expression of GLUT4. In this study, CHO-K1 cells stably overexpressing human c-myc-labelled GLUT4 (CHO-GLUT4) were used to examine glucose transport and GLUT4 translocation to the plasma membrane in response to stimulation by insulin or β-adrenoceptor agonists in cells expressing the wild-type (wt) human β_2-adrenoceptor, truncated or mutant β_2-adrenoceptors. Expression of truncated β_2-adrenoceptors decreased agonist-stimulated glucose uptake, indicating that the C-terminus is important for this response, most likely due to interactions with GRK. In addition, the β_2-adrenoceptor (–)GRK mutant, but not the β_2-adrenoceptor (–)PKA mutant, displayed a marked reduction in GLUT4 translocation compared with wt receptors. βARKct, which is used to knock down G$\beta\gamma$ and thereby investigate Gα-independent signalling pathways, inhibited both GLUT4 translocation and glucose uptake following β_2-adrenoceptor stimulation in cells expressing the wt β_2-adrenoceptor. In addition, β_2-adrenoceptor-stimulated GLUT4 translocation was reduced in cells overexpressing kinase-dead GRK2 (K220R). These results show that β_2-adrenoceptor activation increases glucose uptake and translocation of GLUT4 via GRK sites contained in the C-terminal tail of the β_2-adrenoceptor.

Methods

Cell culture

CHO-GLUT4myc cells (CHO-K1 cells stably expressing human GLUT4 tagged with human *c-myc* epitope) were a gift from Dr Kazuhiro Kishi (University of Tokushima, Tokushima, Japan). Cells were grown in Dulbecco's modified Eagle's medium (DMEM)/Hams-F12 medium supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 4500 $mg \cdot L^{-1}$ D-glucose, 2.5 $\mu g \cdot mL^{-1}$ Amphostat B and 80 $\mu g \cdot mL^{-1}$ gentamicin sulphate, in the presence of the selection antibiotic G418 (400 $\mu g \cdot mL^{-1}$) every third passage. CHO-K1 cells were obtained from the American Type Culture Collection. Cells were grown in incubators at 37°C and 5% CO_2, with medium changes performed every second day.

Generation of β_2-adrenoceptor constructs

The wt human β_2-adrenoceptor coding region was generated by PCR on human genomic DNA. The forward primer incorporated a Hind III site and a Kozak translation initiation consensus sequence (5′GTAAGCTTACCTGCCAGACTG CGCACCATGG3′) and the reverse primer included the β_2-adrenoceptor termination codon and an Xba I site (5′GGGTCTAGAGGAGTAGAAAAACTGCTTTACAGC3′). The 1295 base pair β_2-adrenoceptor PCR fragment was digested with Hind III and Xba I and ligated into the mammalian expression vector pcDNA3.1+. Constructs encoding truncated β_2-adrenoceptors were made by replacing a 742 bp Kpn I-Xba I cassette from the wt sequence with PCR fragments incorporating the coding sequences of the altered C-termini. The forward primer for both constructs was immediately upstream of a Kpn I site present in the wt β_2-adrenoceptor cDNA (5′ CTTACCTCCTTCTTGCCCATTCAGATGC 3′). The reverse primer for β_2-adrenoceptor t344 was 5′ GCT CTAGACGATTACCTGCGCAGGCACAGAAGC 3′, and for

Table 1
Oligonucleotides used as PCR primers

Primers	Strand	Length	Sequence (5′ → 3′)	T_m (°C)	Expected product size (bp)
GRK2	Forward	28	GGAACTGTACCGCAACTTTCCTCTCACC	70	494
	Reverse	25	GCTTGTTCTTCATCTTGGGCACTCG	69	
GRK3	Forward	25	CAACACGGGGTGTTCTCTGAGAAGG	69	519
	Reverse	27	GTCAATGCCCTTGAAGAAGATGTGCTC	69	
GRK4	Forward	27	TGCTGTGGCTTGGAAGATTTACAGAGG	69	477
	Reverse	33	GCTGAAGTTTATATCCTTGAATATTGGGTGCTG	69	
GRK5	Forward	25	CTCCAAAGTCCCCAGTCTTCATTGC	68	414
	Reverse	25	GGTTGACCACAAACTGGCTGTTGAC	68	
GRK6	Forward	21	AGGTGGAGCGGCTGGTCAAGG	70	414
	Reverse	25	GAACAGACCCATCCAGCCCAAAGAC	70	

β_2-adrenoceptor t349 was 5′ AGTCTAGATTCCCTTAGGCC TTCAAAGAAGACC 3′. Following PCR, each fragment was digested with Kpn I and Xba I and ligated into wt β_2-adrenoceptor plasmid from which the corresponding Kpn I-Xba I fragment had been removed. The complete wt and truncated β_2-adrenoceptor inserts and junctions with pcDNA3.1 were checked by DNA sequencing on both strands (Micromon, Monash University, Vic, Australia). β_2-Adrenoceptor(–)PKA, β_2-adrenoceptor(–)GRK and βARKct were gifts from Prof Robert J. Lefkowitz (Duke University Medical Center, Durham, NC, USA). In the β_2-adrenoceptor (–)PKA mutant, S^{261}/S^{262} and S^{345}/S^{346} are replaced by alanine residues. The β_2-adrenoceptor(–)GRK retains the PKA phosphorylation sites, but the remaining 11 C-terminal tail serine and threonine residues are all replaced by alanine residues (Hausdorff *et al.*, 1989).

Transfection of CHO-K1 cells

CHO-GLUT4myc cells were plated at 10^5 cells cm^{-2} the night before transfection and maintained in normal growth medium. The following day, the medium was removed and cells washed once with HEPES buffered saline (150 mM NaCl, 20 mM HEPES pH 7.0). Transfection solution containing pcDNA3.1 plasmid constructs (100 ng cm^{-2} human wt β_2-adrenoceptor, or truncated t349β_2-adrenoceptor, t344β_2-adrenoceptor, β_2-adrenoceptor(–)GRK and β_2-adrenoceptor(–)PKA), and Lipofectamine (plasmid to Lipofectamine ratio approximately 1:3) in phenol red-free Opti-MEM (Invitrogen, Carlsbad CA, USA) was added to the cells. For βARKct and GRK overexpression experiments, cells were transfected with 100 ng wt β_2-adrenoceptor cm^{-2} together with 500 ng βARKct, 100 ng GRKwt or 100 ng GRK K220R. Cells were incubated for 4 h at 37°C and 8% CO_2, transfection medium removed and replaced by normal growth medium. Cells were maintained under normal growth conditions, trypsinized and transferred to plates approximately 32 h after transfection, and used for experiments at least 48 h after transfection.

Reverse transcription-PCR (RT-PCR)

RNA was extracted from CHO-GLUT4myc cells using Trizol, according to the manufacturer's instructions (Invitrogen). RNA was also extracted from the brain, heart, lung, testis and thymus from a 6 month old male FVB/N mouse killed by cervical dislocation (ethics approval from the Monash University Animal Ethics Committee). The yield and quality of RNA were assessed by measurement of absorbance at 260 and 280 nm and electrophoresis on 1% agarose gels. cDNAs were synthesized by reverse transcription of 1 µg of each total RNA using oligo (dT_{15}) as previously described (Roberts *et al.*, 1999) PCR amplification was carried out on cDNA equivalent to 100 ng of starting RNA using intron-spanning primers for GRK2, GRK3, GRK4, GRK5 and GRK6 (Table 1) to yield expected products of 494, 519, 477, 414 and 414 base pairs respectively. For all GRK PCR, PCR mixes (total volume 20 µL) contained 0.5 U Platinum Pfx DNA polymerase (Invitrogen), 1 × Pfx amplification buffer, 130 $\mu mol \cdot L^{-1}$ dNTPs, 1.5 $mmol \cdot L^{-1}$ $MgSO_4$, 5.8 pmol forward and 5.8 pmol reverse primer. GRK2, 3 and 4 PCR mixes contained 1 × PCR enhancer solution, GRK5 PCR mixes contained 2 × PCR enhancer solution and GRK6 PCR mixes contained 3 × PCR enhancer solution (Invitrogen). The annealing temperature was 60°C for all reactions and 28 cycles performed for GRK2 and GRK5 PCR, 30 cycles performed for GRK3 and 6 PCR, and 32 cycles performed for GRK4 PCR. Conditions were based on experiments performed to optimize cycle numbers and enhancer concentration using cDNA derived from control mouse tissues. Following amplification on a MWG-Biotech Primus^{96} Plus PCR machine, products were electrophoresed on 1.3% agarose gels and images captured digitally using a Typhoon TRIO imaging system (GE Healthcare, Piscataway, NJ, USA).

Glucose uptake experiments

Cells were plated at 1.9×10^5 cells per well the night before the experiment in 24-well plates and maintained in 10% FBS media overnight. The medium was replaced in the morning (by serum-free medium) for at least 4 h, before the medium

was replaced again by serum-free medium and drugs added for 2 h at 37°C. After this, cells were washed in warm PBS, glucose-free medium was added, and drugs were re-added for 45 min, after which 2-deoxy-[^{3}H]-D-glucose was added for 15 min. Reactions were terminated by washing twice in ice-cold PBS, cells were digested (0.2 M NaOH, 1 h, 60°C), and samples were transferred to scintillation vials with scintilant and radioactivity counted. When inhibitors were used, the time indicated with the results represents the time cells were pre-equilibrated with the inhibitors before agonists were added. All experiments were performed in duplicate–quadruplicate with *n* referring to the number of independent experiments performed.

cAMP accumulation assay

Cells were plated at 3.6×10^5 cells per well in 12-well plates the night before experiment. Cells were deprived of serum for 4 h, then treated with agonists for 30 min in serum-free DMEM/Ham's F-12 containing 2 mM IBMX to prevent the breakdown of cAMP. The medium was aspirated and reactions terminated by the addition of 70% ethanol containing 4 mM EDTA. cAMP was assayed using the Amersham [^{3}H]-cAMP kit according to the manufacturer's protocol. All experiments were performed in duplicate with *n* referring to the number of independent experiments performed.

When performing concentration-response curves to isoprenaline in the CHO-wtβ_2-adrenoceptor-GLUT4, CHO-t349β_2-adrenoceptor-GLUT4 and CHO-t344β_2-adrenoceptor-GLUT4 cells, cAMP was measured using the Perkin–Elmer αScreen kit. Cells were plated in 96-well plates (20 000 cells per well) and deprived of serum overnight before the cAMP assay was carried out as previously described following stimulation of cells with isoprenaline for 30 min in the presence of 1 mM IBMX (Sato *et al.*, 2008). In each experiment, 100 μM forskolin was used as a positive control. All experiments were performed in duplicate with *n* referring to the number of independent experiments performed. It should be noted that the cAMP levels produced in this set of experiments differ from that produced with the data sets performed using the Amersham [^{3}H]-cAMP kit due to differences in cell density per number and the sensitivity of the assays used. However the isoprenaline responses relative to 100 μM forskolin were similar in both sets of experiments (data not shown).

SDS-PAGE/Western immunoblotting

Cells were grown in 12-well plates at 1×10^5 per well in DMEM/Ham's F-12 containing 0.5% FBS for 2 days, and the medium replaced 2 h before the experiment. For PKB detection, immunoblotting was performed as described previously (Lindquist *et al.*, 2000). Briefly, cells were stimulated for various time points as indicated with the data (5–180 min). Samples were harvested in prewarmed (65°C) SDS buffer containing 50 mM dithiothreitol, then sonicated, boiled for 3 min, and separated on 10% acrylamide gels for 2 h at 100 V. The proteins were transferred to PVDF membranes and probed with primary antibodies, total PKB or pPKB s473 (Cell Signaling, Beverley, CA, USA) diluted 1:2000 overnight at 4°C and detected using a secondary antibody [horseradish peroxidase (HRP)-linked anti-rabbit IgG] diluted 1:2000 for 1 h at room temperature. Proteins were detected by enhanced chemiluminescence and exposure to Hyperfilm ECL film.

For GLUT4 and GRK2/3 detection, cells were washed with ice-cold PBS, lysed by the addition of radio immunoprecipitation assay buffer (PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, and 1 $\mu g \cdot mL^{-1}$ aprotinin) for 15 min at 4°C, cells scraped and samples transferred to microcentrifuge tubes and incubated on ice for 45 min. Samples were centrifuged (10 000× *g*, 10 min, 4°C) and the supernatant transferred to new tubes and kept for future use at –20°C. Protein levels were measured (Lowry *et al.*, 1951) and equal amounts of protein (50 μg) separated on 10% acrylamide gels for 2 h at 100 V. The proteins were transferred to PVDF membranes and probed with primary antibodies, GRK2/3 (1:200 or 1:400 dilution in blocking buffer (5% nonfat milk powder in tris buffered saline with Tween) 1 h at room temperature, Santa Cruz Biotechnology, Santa Cruz, CA, USA), or GLUT4 (1:2000 dilution in blocking buffer overnight at 4°C, Abcam, Cambridge, UK) and detected using a secondary antibody (HRP-linked anti-rabbit IgG) diluted 1:2000 for 1 h at room temperature in blocking buffer. Proteins were detected by enhanced chemiluminescence and exposure to Hyperfilm ECL film.

Immunocytochemistry for detection of GLUT4 translocation

Cells were plated at 1.9×10^5 cells per well in 4-well culture chamber slides (BD Biosciences, Franklin Lakes, NJ, USA) the night before transfection and maintained in normal growth medium for 48 h. After 3 h of insulin or isoprenaline stimulation, cells were fixed for 20 min with 4% formaldehyde in PBS, and quenched with 50 mM glycine in PBS for 10 min. Cells were blocked overnight at 4°C with 5% BSA in PBS and incubated with Myc-tag primary antibody solution (1:200 dilution in 1.5% BSA in PBS) for 1 h at room temperature followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (1:500 dilution, 1.5% BSA in PBS) for 1 h. Slides were mounted with ProLong Gold antifade reagent (Invitrogen). Images were observed in a Leica DMLB epifluorescence microscope (excitation 450–490 nm, dichroic mirror 510 nm, emission 515–535 nm). Images were acquired using a DC350F camera with IM500 software (Leica Microsystems AB, Kista, Sweden). Quantification of immunocytochemistry images was performed by using ImageJ program (National Institutes of Health, Bethesda, MD, USA).

Whole cell [^{3}H]-CGP12177A radioligand binding assay

Radioligand binding studies were performed as described previously (Yamamoto *et al.*, 2007). Data were analysed using non-linear curve fitting (Graph Pad Prism, San Diego, CA, USA) and a one-site model to determine the concentration of ligand required to occupy 50% of the binding sites (K_D) and maximal binding (B_{max}) values.

Statistical analysis and nomenclature

Results were analysed using GraphPad Prism v5 (GraphPad). Significance was determined using Student's *t*-test, or two-way ANOVA; $P < 0.05$ was considered significant. All drug/molecular target nomenclature conforms to British Journal of

Pharmacology's Guide to Receptors and Channels (Alexander *et al.*, 2011).

Drugs and reagents

The βARKct construct was a gift from Prof RJ Lefkowitz (Duke University Medical Centre, Durham, NC, USA). The GRK K220R and GRKwt constructs were kindly provided by Prof JL Benovic (Thomas Jefferson University, Department of Biochemistry and Molecular Biology, Philadelphia, PA, USA). Zinterol hydrochloride was a gift from Bristol–Myers Squibb (Noble Park, Vic, Australia). Drugs and reagents were obtained as follows: DMEM/Hams-F12, fetal bovine serum, L-glutamine (HyClone, Logan, UT, USA); Amphostat B, gentamicin, geneticin (G418), Lipofectamine™ 2000 reagent, phenol red-free Opti-MEM (Invitrogen); [^{3}H]-2-deoxy-D-glucose (specific activity 9.5–12 Ci·mmol^{-1}), [^{3}H]-CGP12177A (specific activity 50 Ci·mmol^{-1}) (Perkin Elmer, Waltham, MA, USA); 8-bromo-cAMP, isobutyl-methylxanthine(–)-isoprenaline, forskolin (Sigma Chemical Co., St. Louis, MO, USA); [^{3}H] cAMP kit (Amersham Biosciences, Piscataway, NJ, USA); insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark). Antibodies were obtained from Cell Signaling, Santa-Cruz Biotechnology or Abcam as specified.

Results

β_2-Adrenoceptor agonist-mediated glucose uptake in CHO-K1 cells requires expression of both GLUT4 and β_2-adrenoceptors

The non-selective β-adrenoceptor agonist isoprenaline and the β_2-adrenoceptor agonist zinterol did not significantly increase glucose uptake in CHO-K1 cells (CHO-control) (Figure 1A), in CHO-K1 cells transfected with human β_2-adrenoceptor alone (CHO-wtβ_2-adrenoceptor) (Figure 1B), or in CHO-K1 cells stably transfected with human GLUT4myc alone (CHO-GLUT4) (Figure 1C). However, in CHO-GLUT4 cells transfected with the β_2-adrenoceptor (CHO-wtβ_2-adrenoceptor-GLUT4), isoprenaline and zinterol significantly increased glucose uptake by 48.9 ± 2.6% ($P < 0.01$, $n = 3$) and 45.3 ± 8.0% over basal ($P < 0.01$, $n = 3$) respectively (Figure 1D). Concentration-response curves performed with zinterol in CHO-wtβ_2-adrenoceptor-GLUT4 cells following 1 h [negative logarithm of the effective concentration 50% (pEC_{50}) 7.9 ± 1.1; maximum response 15 ± 5% over basal; $n = 5$] or 3 h (pEC_{50} 8.9 ± 0.7; maximum response 32 ± 5% over basal; $n = 5$) stimulation shows that responses to zinterol are time- and concentration-dependent ($P < 0.01$, $n = 5$, two-way ANOVA, 3 h) (Figure 1E). There was no detectable binding of [^{3}H]-CGP12177 to the β_2-adrenoceptor in CHO-K1 cells (data not shown) as opposed to CHO-wtβ_2-adrenoceptor-GLUT4 cells, where the β_2-adrenoceptor was expressed at 211 ± 38 fmol·mg^{-1} protein (pK_D 10.00 ± 0.04; $n = 7$).

Insulin increased glucose uptake by 38.1 ± 8.5% ($P < 0.05$, $n = 4$) and 51.3 ± 0.2% over basal ($P < 0.0001$, $n = 3$) in CHO-GLUT4 and CHO-wtβ_2-adrenoceptor-GLUT4 cells respectively (Figure 1C and D). Insulin also stimulated glucose uptake in cells lacking the GLUT4 transporter, namely CHO-control (31.4 ± 4.9% over basal; $P < 0.05$, $n = 3$; Figure 1A), and CHO-wtβ_2-adrenoceptor cells (25.4 ± 2.1%

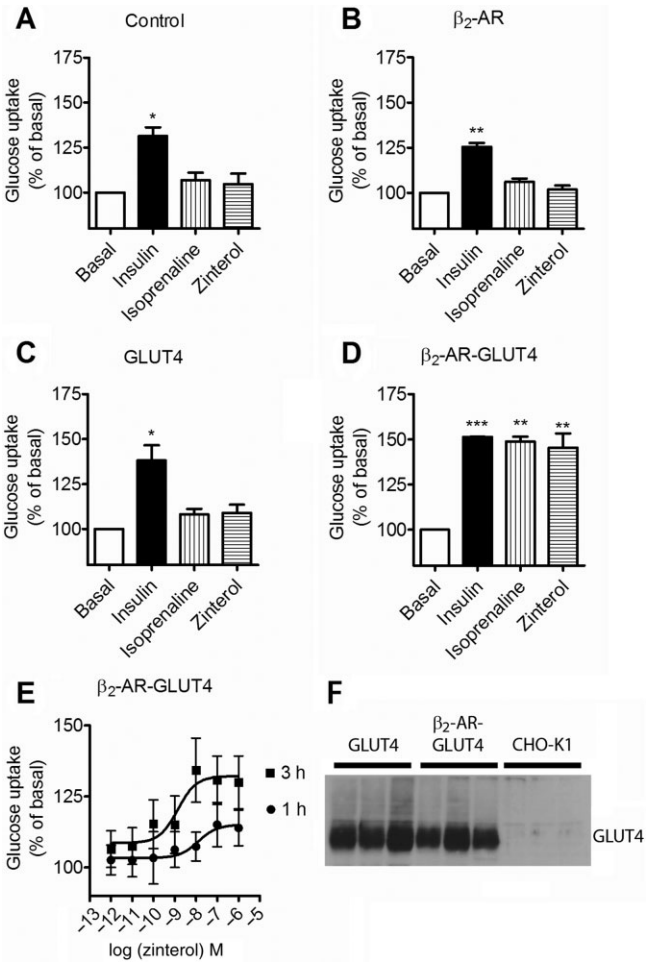

Figure 1

Glucose uptake in response to insulin (1 μM), the non-selective β-adrenoceptor agonist isoprenaline (1 μM), the β_2-adrenoceptor agonist zinterol (1 μM) in (A) CHO-K1 (Control), (B) CHO-K1 transfected with human wt β_2-adrenoceptors (β_2-AR), (C) CHO-K1 transfected with human GLUT4myc (GLUT4), (D) CHO-K1 transfected with human wt β_2-adrenoceptors and GLUT4myc (β_2-AR-GLUT4), (E) Concentration-response curve for stimulation by zinterol for 1 h and 3 h (■) ($n = 5$ experiments in duplicate). Histograms represent means ± SEM of 3–4 experiments performed in duplicate. Asterisks represent statistical difference as analysed by Student's paired *t*-test between basal and treated cells (*$P < 0.05$, **$P < 0.01$ ***$P < 0.001$). (F) Immunoblot of GLUT4 in CHO-K1, CHO-GLUT4myc and CHO-GLUT4myc-wtβ_2-adrenoceptor cells ($n = 3$).

$P < 0.01$, $n = 3$; Figure 1B). In CHO-K1 cells overexpressing the human insulin receptor, insulin promotes a twofold increase in glucose uptake via translocation of GLUT1 transporters (Hara *et al.*, 1994). All of our cells express endogenous levels of insulin receptor, estimated to be 100 fmol·mg^{-1} protein (White *et al.*, 1987). While overexpression of GLUT4 would be expected to produce a substantial increase in insulin-stimulated glucose uptake, the maximal response may be limited by the low abundance of insulin receptors in CHO-K1 cells. There was no detectable GLUT4 protein expression in CHO-K1 cells as opposed to CHO-GLUT4 and CHO-wtβ_2-adrenoceptor-GLUT4 cells (Figure 1F).

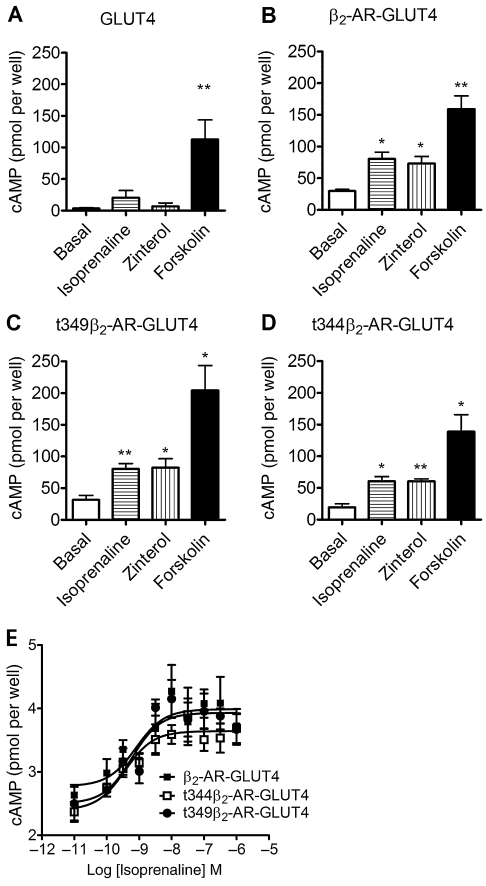

Figure 2

cAMP levels in response to the non-selective β-adrenoceptor agonist isoprenaline (1 μM), the β_2-adrenoceptor agonist zinterol (1 μM) or forskolin 10 μM in (A) CHO-GLUT4myc (GLUT4), (B) human wtβ_2-adrenoceptor transfected in CHO-GLUT4myc (β_2-AR-GLUT4), (C) human truncated t349β_2-adrenoceptor transfected in CHO-GLUT4myc (t349β_2-AR-GLUT4), (D) human truncated t344β_2-adrenoceptor transfected in CHO-GLUT4myc (t344β_2-AR-GLUT4). (E) Concentration-response curve for isoprenaline-stimulated cAMP accumulation in CHO-wtβ_2-adrenoceptor-GLUT4, CHO-t349β_2-adrenoceptor-GLUT4 and CHO-t344β_2-adrenoceptor-GLUT4 cells [note that the results in Figure 2E were measured using a different cAMP kit (as discussed in the Methods section), hence the difference in the relative amounts of cAMP produced]. Histograms represent means ± SEM of 3–5 experiments performed in duplicate, and each point for the concentration-response curve shows mean ± SEM of four experiments performed in duplicate. Asterisks represent statistical difference as analysed by Student's paired *t*-test between basal and treated cells (*$P < 0.05$, **$P < 0.01$).

β_2-Adrenoceptor stimulation increases cAMP levels in CHO-GLUT4 β_2-adrenoceptor cells

In CHO-GLUT4 cells, forskolin (112.6 ± 31.0 pmol per well, $P < 0.05$, $n = 4$), but not isoprenaline (1 μM; 20.2 ± 11.6 pmol per well, $n = 4$) or zinterol (1 μM; 6.6 ± 5.7 pmol per well, $n = 4$) (Figure 2A) increased cAMP levels (basal 3.5 ± 1.3 pmol per well). In contrast, cAMP levels were significantly increased in CHO-wtβ_2-adrenoceptor-GLUT4 cells by isoprenaline (80.6 ± 10.7 pmol per well, $P < 0.05$, $n = 4$) and zinterol (73.3 ± 11.1 pmol per well, $P < 0.05$, $n = 4$) compared with basal (29.7 ± 2.5 pmol per well, $n = 4$) (Figure 2B). Basal levels of cAMP in CHO-wtβ_2-adrenoceptor-GLUT4 cells were significantly higher than in CHO-GLUT4 cells ($P < 0.001$, $n = 4$, Student's unpaired *t*-test). This effect has been reported previously (Nakahara *et al.*, 2004), and may depend on receptor number and constitutive activity of β_2-adrenoceptors.

The primary sites for GRK phosphorylation are S^{355}, S^{356} and S^{364}, located in the β_2-adrenoceptor C-terminal tail (Vaughan *et al.*, 2006; Krasel *et al.*, 2008). We expressed two truncated receptors (t344β_2-adrenoceptor and t349β_2-adrenoceptor) that lack GRK sites; t344β_2-adrenoceptor also lacks the PKA consensus sequence $R^{343}RSS^{346}$. The truncated receptors retain helix 8 that is critical for functional coupling to the Gs/cAMP signal transduction pathway (O'Dowd *et al.*, 1989; Liggett *et al.*, 1989a,b; Mouillac *et al.*, 1992). Before testing whether truncation of the β_2-adrenoceptor affects agonist-stimulated glucose uptake, we verified that both the t349β_2-adrenoceptor and t344β_2-adrenoceptor were functional in CHO-GLUT4 cells by measuring cAMP accumulation. cAMP levels in response to isoprenaline (80.0 ± 8.8 pmol per well $n = 4$) and zinterol (82.3 ± 14.1 pmol per well $n = 4$) in CHO-t349β_2-adrenoceptor-GLUT4 (Figure 2C) or isoprenaline (60.4 ± 7.5 pmol per well $n = 4$) and zinterol (60.2 ± 4.1 pmol per well $n = 4$) in CHO-t344β_2-adrenoceptor-GLUT4 cells (Figure 2D) were not significantly different from those in control CHO-wtβ_2-adrenoceptor-GLUT4 (isoprenaline 80.6 ± 10.5 pmol per well $n = 4$, zinterol 73.3 ± 11.1 pmol per well $n = 4$; Student's unpaired *t*-test). Concentration-response curves to isoprenaline in the CHO-wtβ_2-adrenoceptor-GLUT4 (pEC_{50} 9.10 ± 0.4, $n = 4$), CHO-t349β_2-adrenoceptor-GLUT4 (pEC_{50} 9.23 ± 0.3, $n = 4$) and CHO-t344β_2-adrenoceptor-GLUT4 (pEC_{50} 9.44 ± 0.3, $n = 4$) cells showed no difference in the ability of isoprenaline to increase cAMP with respect to maximal responses produced or pEC_{50} values (Figure 2E). This suggests that the ability of t344- and t349-β_2-adrenoceptors to signal through Gαs is not disrupted by truncation of the C-terminus.

β_2-Adrenoceptor truncation leads to decreased glucose uptake

In contrast to cAMP accumulation, isoprenaline- and zinterol-stimulated glucose uptake were significantly reduced in both CHO-t349β_2-adrenoceptor-GLUT4 and CHO-t344β_2-adrenoceptor-GLUT4 compared with control CHO-wtβ_2-adrenoceptor-GLUT4 (Figure 3). Isoprenaline-stimulated glucose uptake was reduced by 48% in CHO-t349β_2-adrenoceptor-GLUT4 (Student's paired *t*-test **$P < 0.01$) and 58% in CHO-t344β_2-adrenoceptor-GLUT4 cells, respectively (Student's paired *t*-test *$P < 0.05$). Similarly, zinterol-stimulated glucose uptake was reduced by 38% and 51% in CHO-t349β_2-adrenoceptor-GLUT4 (Student's paired *t*-test not significant $P = 0.08$) or -t344β_2-adrenoceptor-GLUT4 (Student's paired *t*-test ***$P < 0.001$), respectively. Responses to insulin were not affected by truncation of the β_2-adrenoceptor C-terminus. In addition, glucose uptake stimulated by the cAMP analogue 8-bromo-cAMP was not significantly different in cells expressing either the wt or truncated β_2-adrenoceptors (Figure 3). It should be noted that basal glucose uptake was unaffected by truncation of the β_2-adrenoceptor (39.06 ± 1.7 dpm·μg^{-1} protein in CHO-wtβ_2-adrenoceptor-GLUT4 cells, 41.01 ± 0.8 dpm·μg^{-1}

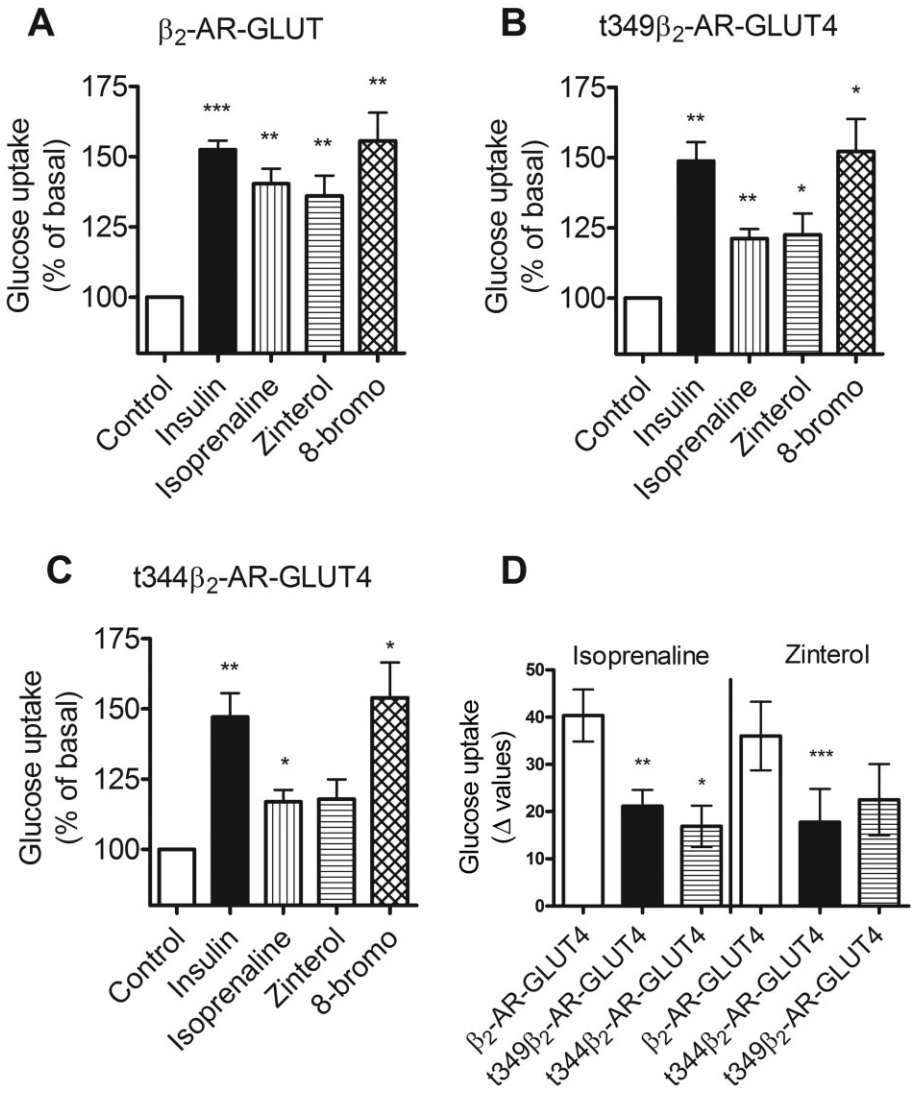

Figure 3

Glucose uptake in response to insulin (1 μM), the non-selective β-adrenoceptor agonist isoprenaline (1 μM), the β_2-adrenoceptor agonist zinterol (1 μM), cAMP analogue 8-bromo-cAMP (1 mM) in (A) human wtβ_2-adrenoceptor transfected in CHO-GLUT4myc (β_2-AR-GLUT4), (B) human truncated t349β_2-adrenoceptor transfected in CHO-GLUT4myc (t349β_2-AR-GLUT4), (C) human truncated t344β_2-adrenoceptor transfected in CHO-GLUT4myc (t344β_2-AR-GLUT4). (D) Isoprenaline and zinterol induced glucose uptake in wtβ_2-adrenoceptor and truncated t349β_2-adrenoceptor and t344β_2-adrenoceptor (Δ values). Histograms represent means ± SEM of 5 experiments performed in duplicate. Asterisks represent statistical difference as analysed by Student's paired *t*-test between control and treated samples (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

protein in CHO-t344β_2-adrenoceptor-GLUT4 cells and 41.42 ± 2.9 dpm·μg^{-1} protein in CHO-t349β_2-adrenoceptor-GLUT4 cells). The reduced agonist-stimulated glucose uptake in cells expressing truncated receptors suggests that the signalling pathway is at least partially dependent on the β_2-adrenoceptor C-terminus.

Effect of βARKct and PTX on β_2-adrenoceptor-mediated glucose uptake

Interaction of GRK2 and GRK3 with Gβγ (and also PIP_2) is governed by a pleckstrin homology domain in the C-terminus. This region (βARKct) competitively inhibits GRK2 (and presumably GRK3) by sequestering Gβγ subunits, preventing kinase recruitment to activated receptors (Koch *et al.*, 1993; Pitcher *et al.*, 1995). βARKct sequestration of Gβγ is widely used to knock down Gβγ and thereby investigate its role in Gα-independent signalling pathways. To examine the potential role of β_2-adrenoceptor phosphorylation by GRKs, CHO-GLUT4 cells were transfected with wtβ_2-adrenoceptor alone, or with βARKct, and glucose uptake measured (Figure 4). Immunoblotting with a GRK2/3 antibody raised against amino acids 468–689 of the C-terminus of the human GRK2 (of which βARKct comprises the last 194 amino acids) verified overexpression of βARKct in CHO-wtβ_2-adrenoceptor-GLUT4 cells (Figure 4A). The level of βARKct was extremely high in the overexpressing cells, so we also

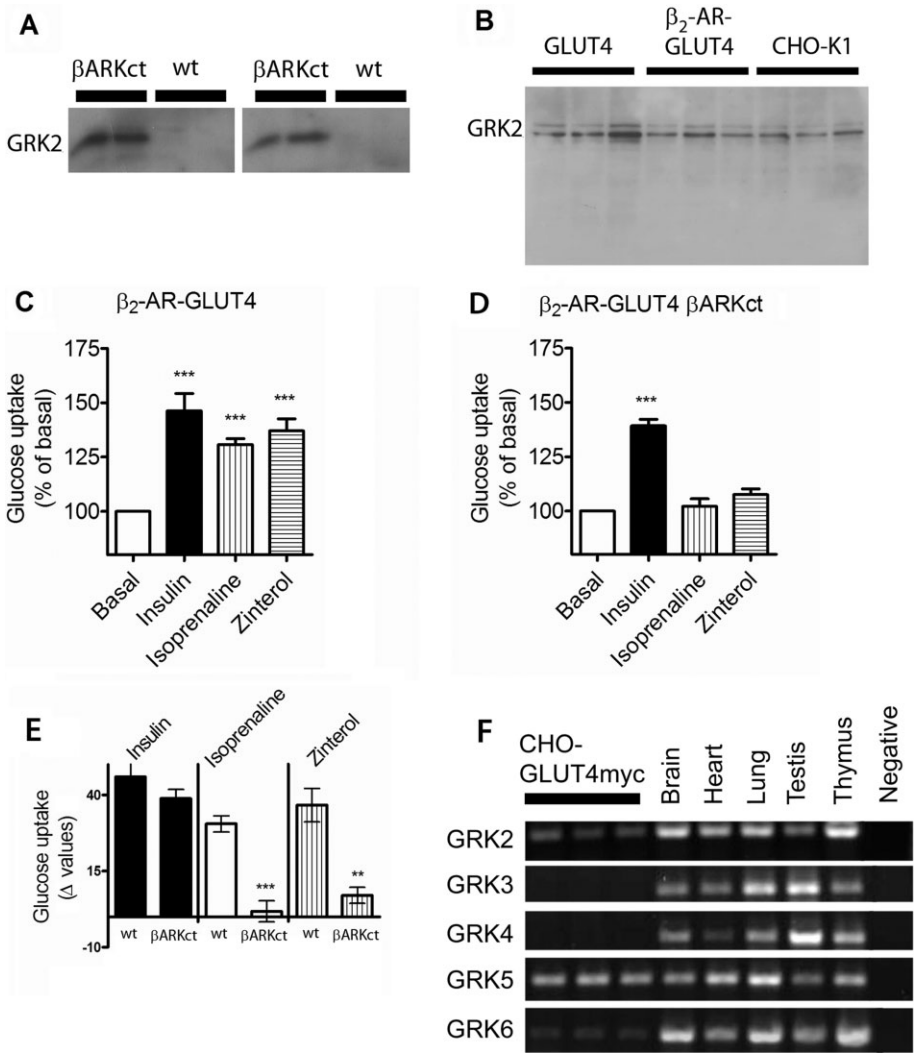

Figure 4

(A) Immunoblot of GRK2 in βARKct-β_2-adrenoceptor-GLUT4 and β_2-adrenoceptor-GLUT4 cells (n = 4) and (B) immunoblot of GRK2 in CHO-GLUT4, CHO-wtβ_2-adrenoceptor-GLUT4 and CHO-K1 cells (n = 3). Glucose uptake in response to insulin (1 μM), the non-selective β-adrenoceptor agonist isoprenaline (1 μM), the β_2-adrenoceptor agonist zinterol (1 μM) in (C) human wtβ_2-adrenoceptor transfected in CHO-GLUT4myc (β_2-AR-GLUT4), (D) βARKct and human wtβ_2-adrenoceptor transfected in CHO-GLUT4myc (βARKct-β_2-AR-GLUT4). (E) Insulin-, isoprenaline- and zintrol-stimulated glucose uptake with or without βARKct (Δ values). (F) RT-PCR showing the different GRK isoforms GRK2-6 in CHO-GLUT4myc cells. Histograms represent means ± SEM of 11 experiments performed in duplicate. Asterisks represent statistical difference as analysed by Student's paired t-test between control and treated samples (**P < 0.01 ***P < 0.001).

performed separate immunoblotting with a longer exposure time, to show that there are similar levels of endogenous GRK2 protein in CHO-GLUT4, CHO-wtβ_2-adrenoceptor-GLUT4 and CHO-K1 cells (Figure 4B).

Co-transfection of CHO-GLUT4 cells with wtβ_2-adrenoceptor and βARKct abolished glucose uptake to isoprenaline (P < 0.001, n = 11), and substantially reduced that to zinterol (P < 0.01, n = 11) as compared with wtβ_2-adrenoceptor cells (Figure 4C, D, E), and transfection with βARKct did not affect basal glucose uptake levels (25.9 ± 3.8 dpm·μg^{-1} protein control vs. 27.2 ± 1.9 dpm·μg^{-1} protein + βARKct). Co-transfection of βARKct into CHO-GLUT4 cells had no effect on responses to insulin. These changes in responses are not due to alterations in β_2-adrenoceptor receptor number following βARKct transfection (control β_2-adrenoceptor cells B_{max} 227 ± 64 fmol·mg^{-1} protein; pK_D 9.99 ± 0.2; + βARKct B_{max} 186 ± 34 fmol·mg^{-1} protein; pK_D 10.05 ± 0.3; n = 4). These data suggest that β_2-adrenoceptor phosphorylation involving one of the GRK isoforms dependent on Gβγ recruitment, namely GRK2 or GRK3, is a critical step in the glucose uptake response. RT-PCR analysis in CHO-GLUT4 cells identified the presence of mRNA for GRK2, GRK5 and GRK6 with no detectable levels of GRK3 or GRK4 mRNA observed (Figure 4F).

Inhibition by βARKct could, however, indicate an alternative pathway mediated by Gβγ subunits. It is known that Gβγ derived from a heterotrimer with GαoA or Gαi3 dissociates in the presence of activated Gα subunits, whereas Gβγ in

complex with active Gαs does not display significant dissociation (Digby *et al.*, 2006). It was suggested that this difference may explain why Gβγ signalling is generally restricted to heterotrimers containing members of the Gαi/o family. We therefore tested whether β_2-adrenoceptor-mediated glucose uptake is inhibited by pertussis toxin (PTX) that selectively blocks activation of Gi/o α subunits. PTX did not inhibit glucose uptake to isoprenaline or insulin (isoprenaline 49.8 ± 1.6%, isoprenaline + PTX 43.7 ± 2.3%; insulin 48.0 ± 3.5%, insulin + PTX 41.4 ± 5.4% (all % of basal); Figure 5A). In addition, Gβγ signalling promotes PI3 kinase activation, which in turn leads to PKB phosphorylation. In CHO-wtβ_2-adrenoceptor-GLUT4 cells, isoprenaline failed to phosphorylate PKB at any time points examined (5–180 min) as compared with the control insulin (5 and 180 min) (Figure 5B). Additionally in CHO-GLUT4 cells expressing the truncated β_2-adrenoceptors, isoprenaline also failed to phosphorylate PKB (Figure 5C).

GLUT4myc translocation in response to β_2-adrenoceptor stimulation

As glucose uptake to β_2-adrenoceptor stimulation occurred only in cells expressing both the β_2-adrenoceptor and GLUT4, we investigated GLUT4myc translocation following either insulin or isoprenaline treatment. The human GLUT4 construct contains an extracellular Myc epitope that was used as a marker for cell surface GLUT4. Immunocytochemistry was performed with nonpermeabilized cells and only detects GLUT4myc at the plasma membrane. In CHO-wtβ_2-adrenoceptor-GLUT4 cells, insulin and isoprenaline increased GLUT4 fluorescence at the cell surface (Figure 6A). Overexpression of βARKct in CHO-wtβ_2-adrenoceptor-GLUT4 cells abolished isoprenaline-stimulated GLUT4 translocation, but not that to insulin (Figure 6B, C), suggesting that GRK phosphorylation of the β_2-adrenoceptor is important for both GLUT4 translocation and glucose uptake. This finding also indicates that GLUT4 translocation is required for β_2-adrenoceptor-mediated glucose uptake, consistent with our observation that no glucose uptake was observed in cells expressing wt β_2-adrenoceptors but no exogenous GLUT4 (Figure 1). In subsequent experiments, GLUT4 translocation was used as the key endpoint to dissect upstream signalling events required for activation of glucose uptake.

GLUT4 translocation was studied in CHO-wtβ_2-adrenoceptor-GLUT4 cells overexpressing the GRK kinase dead mutant GRK2 K220R compared with wild type GRK2 (GRK2wt). Co-transfection of GRK2 K220R abolished GLUT4 translocation in response to isoprenaline in comparison with GRK2wt or in CHO-wtβ_2-adrenoceptor-GLUT4 cells (Figure 7A, B). Immunoblotting of GRK2 was performed in cells overexpressing GRK2 K220R or GRK2wt, demonstrating enhanced protein levels of GRK2 in comparison with CHO-wtβ_2-adrenoceptor-GLUT4 cells (Figure 7C).

GLUT4myc translocation via β_2-adrenoceptors lacking phosphorylation sites for PKA or GRKs

To investigate the importance of PKA or GRK phosphorylation of the receptor tail on the translocation of GLUT4, β_2-adrenoceptor constructs lacking either the PKA or GRK phosphorylation sites were expressed in CHO-GLUT4myc cells. The β_2-adrenoceptor(–)PKA mutant lacks the $R^{343}RSS^{346}$ and $R^{259}RSS^{262}$ PKA phosphorylation sites, and a β_2-adrenoceptor(–)GRK mutant retains the PKA sites but lacks all 11 of the remaining serine and threonine residues present in the C-terminal tail (Hausdorff *et al.*, 1989). These mutant β_2-adrenoceptors are fully functional with respect to agonist binding and stimulation of cAMP accumulation (Hausdorff *et al.*, 1989). CHO-GLUT4 cells expressing β_2-adrenoceptors

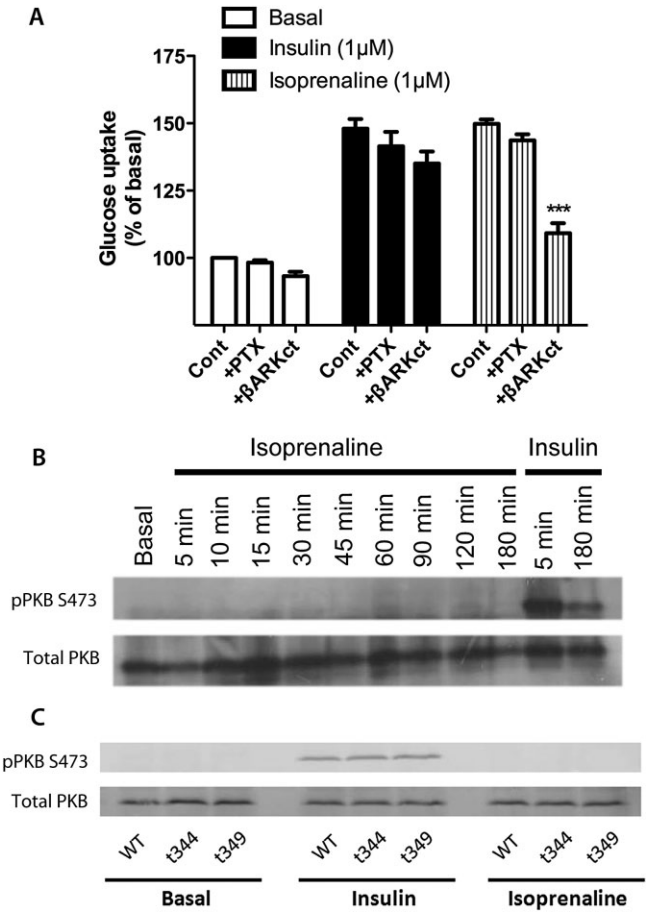

Figure 5

(A) The effect of PTX and βARKct on glucose uptake in response to insulin and isoprenaline in CHO-K1 cells expressing GLUT4 and β_2-adrenoceptors. Pretreatment with PTX (100 ng·mL^{-1}, 16 h) had no effect on glucose uptake responses to insulin or isoprenaline. βARKct (500 ng·cm^{-2}) significantly decreased glucose uptake responses to isoprenaline ($P < 0.0001$). Values represent mean ± SEM from four individual experiments, performed in duplicate. The results are expressed as % basal response. (B) Time-course study of PKB phosphorylation by immunoblotting after isoprenaline (1 μM) treatment for 5, 10, 15, 30, 45, 60, 90, 120 and 180 min and insulin (1 μM) treatment for 5 and 180 min. (C) PKB phosphorylation in response to insulin (1 μM) or isoprenaline (1 μM) in CHO-K1 cells expressing wtβ_2-adrenoceptor-GLUT4, t349β_2-adrenoceptor-GLUT4, or t344β_2-adrenoceptor-GLUT4 after 150 min of treatment. Insulin increased phosphorylation of Ser^{473} of PKB whereas isoprenaline had no effect. Figures are representative of three independent experiments.

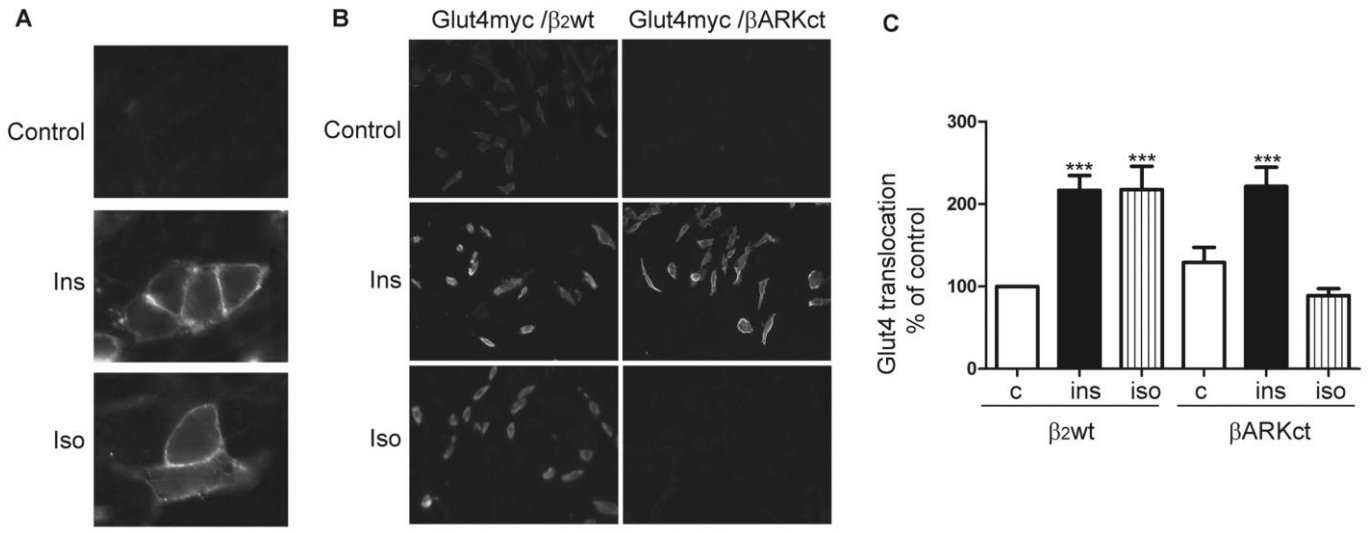

Figure 6

Immunostaining of GLUT4myc in the plasma membrane in (A) human wtβ_2-adrenoceptor transfected in CHO-GLUT4myc cells (wtβ_2-AR-GLUT4). Images show control (untreated cells), cells stimulated with insulin (1 μM) or the non-selective β-adrenoceptor agonist isoprenaline (1 μM) for 3 h. Images were takes at 40 × magnification. In (B) wtβ_2-adrenoceptor transfected in CHO-GLUT4myc cells (wtβ_2-AR-GLUT4) (left panel), and βARKct together with wtβ_2-adrenoceptor transfected in CHO-GLUT4myc (βARKct-wtβ_2-AR-GLUT4) (right panel). Images show control (untreated), cells treated with insulin (1 μM) or isoprenaline (1 μM) for 3 h. Images were taken at 16 × magnification (representative of three separate experiments performed in duplicate). (C) Quantification of immunocytochemistry images by using ImageJ program. Histograms shows means ± SEM of four experiments and the data are expressed as % of control. Asterisks represent statistical significance between wtβ_2-adrenoceptor control and insulin resp. isoprenaline treated cells and in βARKct cells between control and insulin treatment (***$P < 0.001$).

lacking PKA sites were able to translocate GLUT4 in response to isoprenaline to the same extent as wtβ_2-adrenoceptors. However, β_2-adrenoceptors lacking GRK phosphorylation sites showed a substantial reduction in GLUT4 translocation compared with wtβ_2-adrenoceptors (Figure 8), suggesting that GRK phosphorylation of the β_2-adrenoceptor, but not PKA phosphorylation, is important for GLUT4 translocation.

Discussion

Adrenergic stimulation of glucose uptake is potentially an important player in glucose homeostasis as it operates independently of insulin signalling in important glucose-consuming organs such as brown fat and skeletal muscle (Hutchinson *et al.*, 2005, p. 250; 2007, p. 48; Dallner *et al.*, 2006, p. 21). Our previous findings in L6 cells suggested the involvement of cAMP in glucose uptake (Nevzorova *et al.*, 2002, p. 14), but whereas cAMP accumulation showed progressive desensitization over 3 h, glucose uptake was maximal at this time (Nevzorova *et al.*, 2006. p. 388). This suggested that desensitization of β_2-adrenoceptor-Gαs coupling was accompanied by stimulation of an alternative signalling pathway causing glucose uptake, in a manner analogous to activation of MAPK. Consequently, we investigated the role of GRK phosphorylation in β_2-adrenoceptor-mediated glucose uptake, using CHO-GLUT4 cells expressing β_2-adrenoceptors.

We used several approaches, including truncation of the β_2-adrenoceptor C-terminus containing serine and threonine residues that are known GRK phosphorylation sites. Our results show that truncation of the β_2-adrenoceptor tail (t344β_2-adrenoceptor and t349β_2-adrenoceptor) reduces glucose uptake approximately 50% compared with cells expressing wt-β_2-adrenoceptors. This indicates that the receptor C-terminal tail is involved in β_2-adrenoceptor-mediated glucose uptake but the partial inhibition may suggest that other unknown upstream residues in the intracellular loops of the β_2-adrenoceptor, or additional pathways activated by the β_2-adrenoceptor may also be involved in glucose uptake. Coupling of the β_2-adrenoceptor to Gαs promotes activation of adenylyl cyclase and production of cAMP. Although we found that 8-bromo-cAMP produced robust glucose uptake similar to the insulin response, it is unlikely that β_2-adrenoceptor-mediated glucose uptake in our system is via cAMP. 8-Bromo-cAMP is a cell-permeable analogue that is also resistant to degradation by phosphodiesterases (Skogsberg *et al.*, 2008), thus it would be retained in cells over the 3 h time course of the glucose uptake experiments. In the absence of IBMX, cellular cAMP peaks within 90 s, and declines to background levels within 6 min due to PKA-mediated activation of phosphodiesterases, and desensitization of the β_2-adrenoceptor (Violin *et al.*, 2008). The kinetics of glucose uptake, on the other hand, is much slower, with very low responses observed even after 1 h of receptor stimulation. In addition, we found that treatment of CHO-wtβ_2-adrenoceptor-GLUT4 cells with βARKct essentially abolished both GLUT4 translocation and glucose uptake. We therefore focused the remainder of this study on the mechanism by which

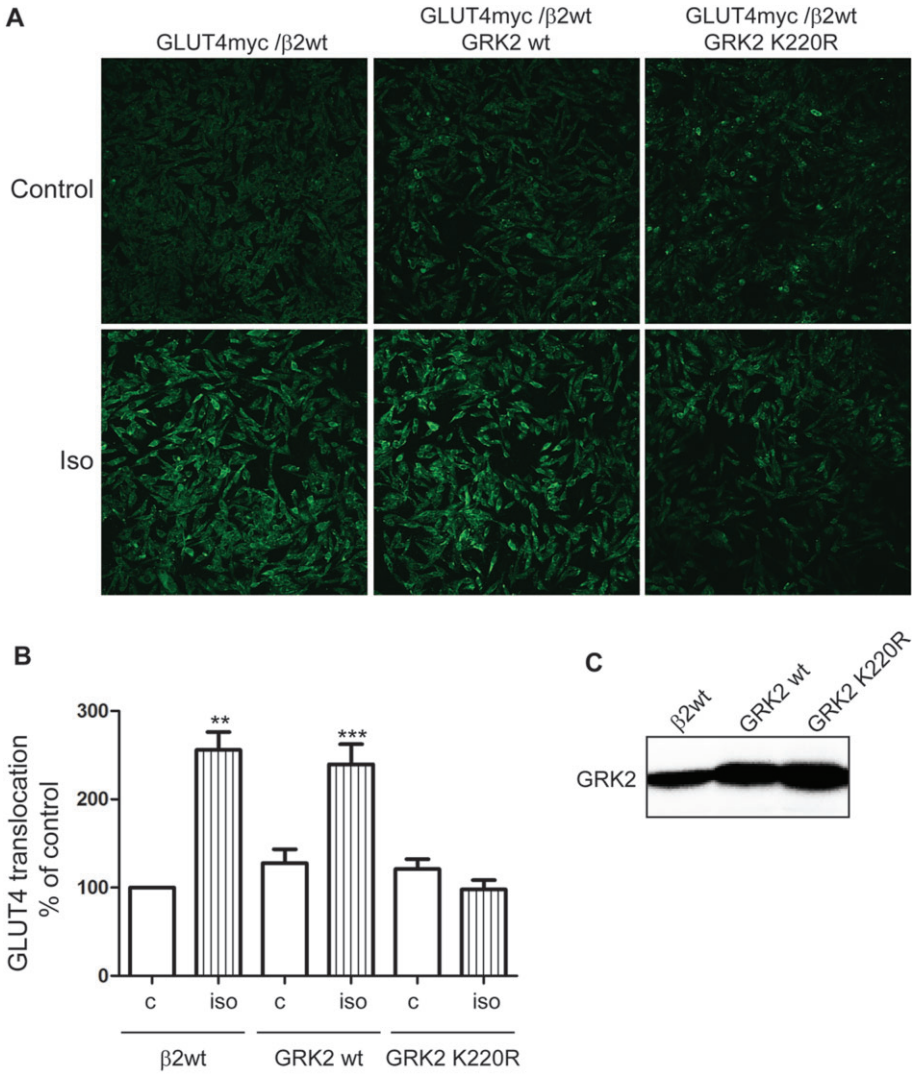

Figure 7

Immunostaining of plasma membrane GLUT4myc in CHO-GLUT4myc-wtβ_2-adrenoceptor cells transfected with human GRK wt or GRK K220R. (A) Images show untreated cells (control) and cells stimulated with non-selective β-adrenoceptor agonist 1 μM isoprenaline (Iso) for 3 h. Images were taken at 20 × magnification. (B) Image J program was used to perform quantification of immunocytochemistry images and data are expressed as % of control. Histograms shows means ± SEM of three experiments performed in quadruplicate. Asterisks represent statistical significance between control and isoprenaline treatment in wtβ_2-adrenoceptor and GRKwt cells (**P < 0.01, ***P < 0.001). (C) Immunoblot of GRK2 in CHO-GLUT4myc-wtβ_2-adrenoceptor cells transfected with human GRK wt or GRK K220R.

βARKct inhibits GLUT4 translocation in cells expressing the β_2-adrenoceptor.

CHO-K1 cells express GLUT1 at low levels, although this transporter has been reported to have relatively high intrinsic activity in these cells (Harrison *et al.*, 1991). Our results indicate that β_2-adrenoceptor-mediated glucose uptake primarily requires GLUT4 translocation to the plasma membrane as the expression of both β_2-adrenoceptor and GLUT4 were necessary for the response, and immunocytochemical studies demonstrated GLUT4 translocation in response to isoprenaline in CHO-wtβ_2-adrenoceptor-GLUT4 cells, similar to that observed with insulin stimulation. Overexpression of βARKct in CHO-wtβ_2-adrenoceptor-GLUT4 cells inhibited GLUT4 translocation in response to isoprenaline, but not to insulin. We showed that overexpression of dominant negative GRK2 (K220R) also abolished GLUT4 translocation, confirming that βARKct acts via inhibition of GRK2 rather than any alternative mechanisms. We also found that a mutant β_2-adrenoceptor lacking all 11 possible sites of GRK phosphorylation was unable to mediate GLUT4 translocation. Importantly, previous studies showed that β_2-adrenoceptors in which the C-terminal tail Ser/Thr residues have been mutated to Gly/Ala show no impairment of receptor-mediated cAMP responses following receptor activation (Kobilka *et al.*, 1987; O'Dowd *et al.*, 1988). Our data therefore provide further support for the role of GRK2 in promoting GLUT4 translocation by the β_2-adrenoceptor. In addition, the observed inhibition of isoprenaline responses in the presence of cata-

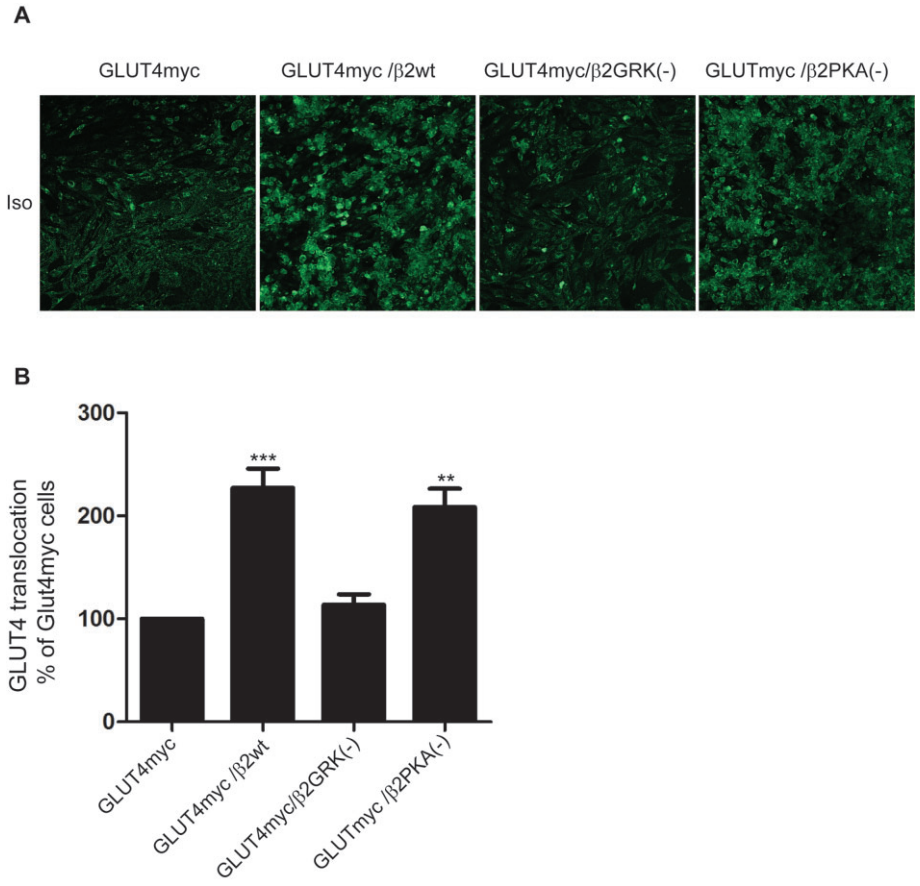

Figure 8

Plasma membrane GLUT4myc immunostaining of CHO-GLUT4myc cells transfected with human wtβ_2-adrenoceptors, GRK(–)β_2-adrenoceptors or PKA(–)β_2-adrenoceptors. (A) Images show cells stimulated with non-selective β-adrenoceptor agonist isoprenaline (1 μM) for 3 h. Images were taken at 10 × magnification. (B) Quantification of immunocytochemistry images was done by using the image J program. Histograms shows % of isoprenaline-stimulated CHO-GLUT4myc cells, means ± SEM of three experiments performed in quadruplicate. Asterisks represent statistical significance between GLUT4myc and wtβ_2-adrenoceptor resp. PKA(–)β_2-adrenoceptor (**P < 0.0, ***P < 0.001).

lytically inactive, dominant-negative GRK2 shows that in our system, it is the GRK2 kinase activity that is important in promoting GLUT4 translocation. This is of interest because GRK2 possesses a regulator of G protein signalling (RGS) domain, distinct from the kinase domain, that specifically inhibits Gαq/11 function (Usui *et al.*, 2004). In 3T3-L1 adipocytes, insulin receptors can phosphorylate Gαq/11, leading to activation of PI3 kinase via cdc42. Usui and co-workers found that overexpression of wild type or kinase-dead GRK2 both suppressed insulin-stimulated GLUT4 translocation, whereas a mutant GRK2 with no RGS domain did not cause any inhibition (Usui *et al.*, 2004). These findings are not consistent with our observation that βARKct had no effect on insulin-stimulated GLUT4 translocation; however, the discrepancy may be a function of cell background, or reflect the relatively low level of insulin receptors in our cells compared with adipocytes. Alternatively, βARKct might not affect this aspect of GRK2 function if the interaction between GRK2 and Gαq/11 were independent of recruitment by Gβγ.

Further studies will be required to determine the pathway linking the β_2-adrenoceptor, GRK2 and GLUT4 translocation. Hara *et al.* (1994) showed that in CHO–K1 cells, expressing the human insulin receptor but not GLUT4, overexpression of a dominant negative mutant of the p85 regulatory subunit of PI3-kinase, that is unable to activate p110 catalytic subunit, blocked glucose uptake and GLUT1 translocation. Likewise, in cells expressing GLUT4, activation of PI3-kinase and downstream PKB represents an obligatory step in GLUT4 translocation and glucose uptake (Wang *et al.*, 1999). Although not the case in all cell types, it seems highly likely that in CHO-K1 cells, insulin promotes GLUT1- or GLUT4-mediated glucose uptake via a common pathway, namely PI3-kinase, PKB activation and translocation of GLUT to the plasma membrane. Insulin stimulated PKB phosphorylation in our CHO-wtβ_2-adrenoceptor-GLUT4 cells, but isoprenaline produced no response. This combined with the lack of GLUT1-mediated glucose uptake in CHO-β_2-adrenoceptor cells indicates that isoprenaline and zinterol stimulate an alternative pathway leading to GLUT4 translocation, rather than the PI3-kinase/PKB pathway. Our findings show that this alternative pathway is downstream of GRK2, suggesting the possible involvement of β-arrestins. Indeed β-arrestin2 has been shown to promote insulin sensitivity; however, this occurred via formation of a complex comprising the activated

insulin receptor, c-Src and PKB, with resulting activation of PKB (Luan *et al.*, 2009). While interaction between the phosphorylated β_2-adrenoceptor and β-arrestin2 may initiate other signalling pathways, we have demonstrated that β_2-adrenoceptor agonists are unable to stimulate PKB phosphorylation, ruling out this particular mechanism.

Our data are consistent with the proposal that isoprenaline-stimulated GLUT4 translocation requires β_2-adrenoceptor-mediated release of Gβγ subunits to facilitate recruitment of GRK2, which phosphorylates target residues in the receptor C-terminal tail. Previous studies showed that βARKct sequesters Gβγ subunits, thereby preventing activation of Gβγ-dependent effectors, but also inhibiting recruitment of the G protein-coupled receptor kinases GRK2 and GRK3 to activated receptors (Koch *et al.*, 1993; Pitcher *et al.*, 1995). The capacity of βARKct to inhibit GRK activity was originally shown using reconstituted phospholipid vesicles containing PIP2 and purified β_2-adrenoceptor, GRK2 and Gβγ (Pitcher *et al.*, 1995). Later work demonstrated that agonist-stimulated translocation of either GRK2 or GRK3 to the plasma membrane of intact cells is dependent upon direct interaction with Gβγ subunits (Daaka *et al.*, 1997). In contrast, GRK5 and GRK6 are constitutively associated with the plasma membrane and do not interact with Gβγ following agonist stimulation (Teli *et al.*, 2005). Our demonstration that isoprenaline-stimulated GLUT4 translocation and glucose uptake are significantly inhibited by βARKct suggests involvement of GRK2 or 3, rather than GRK4-6, although there is convincing evidence that the β_2-adrenoceptor is also phosphorylated by GRK5 and GRK6 (Violin *et al.*, 2006). Our PCR data indicate that CHO-K1 cells express relatively high levels of GRK5 mRNA, with lower levels of GRK2 and GRK6, and no detectable GRK3 or GRK4. It is highly likely that the β_2-adrenoceptor is phosphorylated by GRK5 in CHO-GLUT4 cells, and we cannot discount the possibility that GRK5 and GRK2 are both required for GLUT4 translocation. If GRK5 were the predominant kinase involved in β_2-adrenoceptor phosphorylation, the capacity of both βARKct and kinase-dead GRK2 to abolish GLUT4 translocation would indicate that GRK2 kinase activity is required for phosphorylation of an alternative substrate protein. For instance, there is evidence that GRK2 phosphorylates and thereby activates the actin-binding protein ezrin (Cant and Pitcher, 2005), which is one of three closely related ezrin-radixin-moesin proteins that crosslink cortical actin to the plasma membrane, and is required for both GPCR internalization and remodelling of the actin cytoskeleton (Cant and Pitcher, 2005). Because recruitment of GLUT4-containing vesicles to the plasma membrane also requires cortical actin remodelling (Tong *et al.*, 2001), this mechanism should be considered along with β-arrestin signalling as a possible downstream outcome of β_2-adrenoceptor-mediated GRK2 activation.

In conclusion, our study demonstrates that GRK2 is required for GLUT4 translocation in response to β_2-adrenoceptor agonists. In conjunction with other studies, our findings indicate that stimulation of GLUT4 translocation and glucose uptake by insulin and GPCRs occurs via multiple signalling pathways, and that the predominant mechanisms are highly cell-type dependent. The current findings will facilitate further investigation of the mechanism underlying GRK2-dependent increases in GLUT4 translocation and glucose uptake, and the possible involvement of arrestins or remodelling of the actin cytoskeleton. Potentially, β_2-adrenoceptor agonists that selectively activate this alternative pathway may provide a basis for development of drugs that promote glucose uptake independently of compromised insulin responsiveness in conditions such as Type II diabetes.

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Conflict of interest

None.

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