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ORIGINAL ARTICLE

Role of G protein-coupled receptor kinases (GRKs) in β_2-adrenoceptor-mediated glucose uptake

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Abstract

Truncation of the C-terminal tail of the β_2-AR, transfection of βARKct or over-expression of a kinase-dead GRK mutant reduces isoprenaline-stimulated glucose uptake, indicating that GRK is important for this response. We explored whether phosphorylation of the β_2-AR by GRK2 has a role in glucose uptake or if this response is related to the role of GRK2 as a scaffolding protein. CHO-GLUT4myc cells expressing wild-type and mutant β_2-ARs were generated and receptor affinity for [^{3}H]-CGP12177A and density of binding sites determined together with the affinity of isoprenaline and BRL37344. Following receptor activation by β_2-AR agonists, cAMP accumulation, GLUT4 translocation, [^{3}H]-2-deoxyglucose uptake, and β_2-AR internalization were measured. Bioluminescence resonance energy transfer was used to investigate interactions between β_2-AR and β-arrestin2 or between β_2-AR and GRK2. Glucose uptake after siRNA knockdown or GRK inhibitors was measured in response to β_2-AR agonists. BRL37344 was a poor partial agonist for cAMP generation but displayed similar potency and efficacy to isoprenaline for glucose uptake and GLUT4 translocation. These responses to β_2-AR agonists occurred in CHO-GLUT4myc cells expressing β_2-ARs lacking GRK or GRK/PKA phosphorylation sites as well as in cells expressing the wild-type β_2-AR. However, β_2-ARs lacking phosphorylation sites failed to recruit β-arrestin2 and did not internalize. GRK2 knock-down or GRK2 inhibitors decreased isoprenaline-stimulated glucose uptake in rat L6 skeletal muscle cells. Thus, GRK phosphorylation of the β_2-AR is not associated with isoprenaline- or BRL37344-stimulated glucose uptake. However, GRKs acting as scaffold proteins are important for glucose uptake as GRK2 knock-down or GRK2 inhibition reduces isoprenaline-stimulated glucose uptake.

Abbreviations: AC, adenylyl cyclase; BRET, bioluminescence resonance energy transfer; BRL37344, (±)-(R*,R*)-[4-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]phenoxy]acetic acid sodium hydrate; cAMP, cyclic adenosine monophosphate; CGP12177A, 4-[3-[(1,1-Dimethylethyl)amino]2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one hydrochloride; CHO, Chinese hamster ovary; CMPD101, compound 101; FBS, fetal bovine serum; GLUT4, glucose transporter 4; GRK, G protein-coupled receptor kinase; HEK, Human embryonic kidney; IBMX, 3-isobutyl-1-methylxanthine; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; β_2-AR, β_2-adrenoceptor; β_3-AR, β_3-adrenoceptor.

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KEYWORDS
glucose uptake, GRK2, β_2 adrenoceptor

1 | INTRODUCTION

The increase in the number of people diagnosed with type 2 diabetes increases yearly. Current diabetic medications work primarily by decreasing hepatic glucose production, promoting renal glucose excretion, increasing insulin release/sensitivity, decreasing glucagon release, increasing incretin levels, and/or increasing satiety. There are no diabetic medications that directly target skeletal muscle, despite skeletal muscle insulin resistance being one of the first markers for the development of diabetes. The β_2-adrenoceptor (β_2-AR) has functional roles in skeletal muscle that include regulation of whole-body glucose homeostasis by increasing glucose uptake into skeletal muscle. However, a main limitation of targeting β_2-ARs in skeletal muscle is their undesired effects on the cardiovascular system. Developing a β_2-AR ligand that has preferential effects in skeletal muscle compared to other tissues would be desirable. A recent study[1] has shown the development of a β_2-AR ligand that has anabolic and functional effects on skeletal muscle in vivo with reduced cardiovascular side effects. Hence, the importance in understanding the molecular mechanisms whereby this can occur may open up the avenue for the use of β_2-AR ligands in the treatment of diabetes.

β_2-ARs classically couple to Gαs proteins that activate adenylyl cyclase (AC), leading to increases in intracellular cAMP levels and activation of protein kinase A (PKA), effects that are responsible for many of the cellular responses following β_2-AR activation. One such response, β_2-AR mediated glucose uptake in skeletal muscle, is attenuated by inhibition of PKA/AC and mimicked by cyclic adenosine monophosphate (cAMP) analogs.[2–4] In skeletal muscle, cAMP levels rise rapidly in response to high efficacy β_2-AR agonists such as isoprenaline[5] before cAMP is degraded by phosphodiesterases, whereas β_2-AR mediated glucose uptake by isoprenaline increases more slowly and the effect is sustained for up to 20 h.[5] This suggests that the transient nature of the β_2-AR cAMP response may occur in parallel with an alternative mechanism that maintains increased glucose uptake, highlighting the importance of understanding the molecular mechanisms involved. Transcriptional regulation may also be a factor considering the differences in time courses observed but isoprenaline-mediated glucose uptake in skeletal muscle is independent of both transcription and translation, at least 2 h post-receptor stimulation.[4,5] In addition, partial agonists such as BRL37344 produce only modest cAMP responses yet have similar efficacy to isoprenaline for glucose uptake.

Following activation by full agonists such as isoprenaline, β_2-ARs are rapidly desensitized by G protein-coupled receptor kinase (GRK)-mediated receptor phosphorylation followed by receptor interactions with scaffolding proteins such as β-arrestin, and receptor internalization. However, receptor phosphorylation, interaction with β-arrestins and internalization and desensitization do not occur following activation of β_2-ARs by partial agonists such as BRL37344.[5] GRK[6–9] and PKA-mediated phosphorylation[10–12] of β_2-ARs occurs at serine or threonine residues located within consensus sites in the third intracellular loop and C-terminal tail of the receptor. In human embryonic kidney (HEK) 293 cells, GRK2, 3, 5, and 6 are expressed but their importance for β_2-AR phosphorylation and β-arrestin recruitment is related to their level and sites of expression. GRK2 and 6 are highly expressed with GRK2 being predominantly cytosolic and GRK6 membrane-associated.[13] GRKs are serine/threonine protein kinases that not only have an important role in ligand-occupied receptor phosphorylation but also have interactions with proteins that do not involve phosphorylation. It has been suggested that they should be considered multi-functional proteins.[14,15] Of the 7 GRK isoforms defined to date, GRK2 and GRK5 are the predominant GRK isoforms expressed in skeletal muscle,[16–18] where they are involved in the modulation of skeletal muscle contractile function and physiology.[19] GRK2 also facilitates phosphoinositide 3-kinase (PI3K) translocation to the cell membrane,[20] binds Akt,[21] and phosphorylates insulin receptor substrate 1, an important regulator of insulin sensitivity and glucose uptake, indicating metabolic roles in addition to phosphorylation of GPCRs.

Our previous work has involved investigating the mechanism whereby β_2-ARs increase skeletal muscle glucose uptake.[2–5,22] We and others have shown that activation of β_2-ARs increases glucose uptake in skeletal muscle using cell lines (mouse C_2C_{12}, rat L6 skeletal muscle, human skeletal muscle cells) and isolated skeletal muscle strips, and that β_2-AR agonist administration in vivo improves glucose tolerance in diabetic rats and obese mice.[4] A level of complexity arises from in vivo studies in rodents using BRL37344 as it is a dual rodent β_2–/β_3-AR agonist and its beneficial effects at least in rodents are most likely due to its action at adipocyte β_3-ARs and skeletal muscle β_2-ARs. However, we have shown previously that BRL37344 improves glucose tolerance and increases skeletal muscle glucose uptake ex vivo and in vivo in mice devoid of β_3-ARs,[5] with the focus in this study its actions solely at the β_2-AR. The aim of the current study was to explore the contribution of GRK and PKA phosphorylation in the C-terminal tail of the β_2-AR to cAMP production, glucose uptake and glucose transporter 4 (GLUT4) translocation in response to the full agonist isoprenaline and the partial agonist BRL37344, as well as agonist-promoted β_2-AR internalization.

2 | METHODS

2.1 | Generation of constructs for human β_2-AR

The human β_2-AR and SNAP tagged β_2-AR in pDONR201 entry vectors were obtained from Dr Nick Holliday, Nottingham University. The mutant β_2-ARs in pDONR201 vectors were generated using the

QuickChange site-directed mutagenesis kit according to the manufacturer's instructions. The following variants were produced in addition to the wild-type human β_2-AR: β_2-AR (–)GRKcom with 11 GRK site mutations: β_2-AR (–)GRK/PKA with 15 GRK/PKA site mutations: β_2-AR DALL with 1 mutation in the C-terminal PDZ motif (Figure S1). β_2-AR constructs in pDONR201 were subcloned into either pcDNA6.2/C-EMGRP-DEST, pcDNA3.1/Zeo(+) for SNAP-tagged β_2-ARs, bicistronic for BRET (Figure S2) or pCR3.1 for BRET (Figure S3) vectors.

2.2 | Cell culture

Chinese hamster ovary (CHO) cells and rat skeletal muscle (L6 myoblast) cells were obtained from Dr Kazuhiro Kishi (University of Tokushima, Tokushima, Japan)[23] and Dr Amira Klip, Hospital for Sick Children, Toronto, Canada,[24] respectively. Both stably express GLUT4 protein containing the human c-myc epitope in the first exofacial loop (GLUT4myc). CHO cells were grown in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F12 (DMEM/F12) containing 10% (v/v) fetal bovine serum (FBS) and L6 cells in high glucose DMEM containing 10% FBS. L6 cells grown as myoblasts (undifferentiated cells) were not allowed to grow to more than 70% confluence. To differentiate L6 cells into myotubes, L6 cells were grown to 90% confluence, before differentiation initiated by lowering the FBS content to 2% FBS, with medium change every second day for 7 days. Cells were maintained at 37°C in 5% CO_2. Cell lines are routinely tested for the presence of mycoplasma using the MycoAlert Mycroplasma Detection Kit (Lonza).

2.3 | Generating stable cell lines

CHO-GLUT4myc cells were plated at 50% confluency in T25 flasks. The next day, 2 μg of plasmid carrying each β_2-AR variant was transfected using lipofectamine 2000 according to the manufacturer's instructions. Cells were incubated for 24 h at 37°C in 5% CO_2. Cells were split and maintained in growth medium containing the selection antibiotics; G418 (0.8 mg/mL) and blasticidin (20 μg/mL) for untagged receptor or zeocin (200 μg/mL) for SNAP tagged receptor for 2 weeks. Selection medium was changed every 3–4 days. After selection, cells were re-plated into 96-well plates after diluting to provide a single cell per well. Each well was expanded into a separate colony.

2.4 | Receptor binding

Radioligand binding assays were performed as previously described.[25] Briefly, cells were plated at 1×10^4 cells/well in white clear bottom 96-well microplates. The next day, the medium was removed and cells were incubated in 100 μL of serum-free DMEM/F12 with [^{3}H]-CGP12177A in the absence (total binding) or presence of 10 μM (S)-(–)-propranolol (non-specific binding) whereas for competition binding, cells were incubated with [^{3}H]-CGP12177A (~2 nM) and a range of concentrations of isoprenaline or BRL37344, with non-specific binding determined by 10 μM (–)-propranolol. Incubations were performed at 37°C for 2 h. The assay was terminated by 2 washes in cold PBS, and 100 μL of Microscint 20 (PerkinElmer, MA, USA) added to each well. White plate back and top seals (PerkinElmer, MA, USA) were applied to the plates, incubated overnight at room temperature (20–24°C) and counted on a Topcount NXT at 21°C for 2 min/well. [^{3}H]-CGP12177A concentration was determined by counting 50 μL with 6 mL Microscint 20 (PerkinElmer, MA, USA) and 500 μL of 0.1 M imidazole (Sarstedt, Nümbrecht, Germany) on a TriCarb 2910TR β counter (PerkinElmer, MA, USA) at 21°C for 3 min. Cellular protein was determined following cell lysis in 100 μL 0.4 M NaOH, using the PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA).

2.5 | Cyclic AMP accumulation

cAMP was measured using the LANCE assay (PerkinElmer, MA, USA). L6 cells were differentiated into myotubes prior to experiments then serum-starved for 24 h. Cells were plated at 1×10^4 cells/well in 96-well plates, serum-starved overnight then treated with agonists in stimulation buffer (0.1% BSA [w/v], 5 mM HEPES, 1× Hank's balanced salt solution [HBSS; Gibco], pH 7.4) in the presence of 0.5 mM IBMX for 30 min at 37°C in 5% CO_2. Reactions were terminated by addition of 100% ice cold ethanol that was evaporated at room temperature (20–24°C) and the lysis buffer (0.1% BSA (w/v), 0.3% tween-20 (w/v), 5 mM HEPES, H_2O, pH 7.4) added. About 10 μL of each standard and samples were added to white OptiPlate-384 microplates. About 5 μL of antibody solution (Alexa Fluor® 647-anti cAMP antibody 1:100 in detection buffer – 50 mM HEPES, 10 mM calcium chloride, and 0.35% Triton X-100, pH 7.4) was added and incubated for 30 min at room temperature (20–24°C). About 10 μL of detection mix containing 0.02% LANCE Eu-W8044 labeled streptavidin and 0.07% Biotin cAMP in detection buffer was added and incubated for 2 h at room temperature (20–24°C). cAMP was measured using a Envision plate reader. In each experiment, 100 μM forskolin was used as a positive control and all experiments were performed in duplicate.

2.6 | [^{3}H]-2-deoxyglucose uptake

Cells were plated at 5×10^4 cells/well in 24-well plates and incubated overnight. L6 cells were differentiated into myotubes prior to experiments then serum-starved for 24 h. Serum-free medium was changed before adding drugs for 2 h at 37°C. After 2 washes with pre-warmed PBS, medium was replaced with glucose free medium, and the drugs re-added for 30 min. After incubation with 2-deoxy-[^{3}H]-D-glucose for 15 min, cells were washed three times

with ice cold PBS and digested with 0.2 M NaOH at 60°C for 1 h. Samples were transferred to scintillation vials with liquid scintillation cocktail and radioactivity counted using a TriCarb 2910TR β counter.

2.7 | GLUT4 translocation

GLUT4 translocation assays were performed as previously described.[22] Cells were plated at 1×10^4 cells/well in black clear bottom 96-well microplates 1 day before experiment then incubated with agonists in DMEM/F12 (1:1) supplemented with 2 mM L-glutamine for 2 h at 37°C, 5% CO_2. The reaction was stopped by aspirating the medium, and cells were fixed with 100 μL of 1% paraformaldehyde in PBS for 20 min at room temperature (20–24°C) to limit cell permeabilization, quenched with 100 μL of 1× quench buffer (0.15 M Tris in PBS, pH 8.0) for 10 min at room temperature (20–24°C), and blocked with 5% (w/v) of BSA in PBS for 2 h at room temperature (20–24°C). Cells were washed with PBS before incubation with 50 μL of myc-tag primary rabbit polyclonal antibodies (#2278 Cell Signaling, MA, USA) (1:200 dilution in 1.5% [w/v] BSA in PBS) overnight at 4°C. Next day, cells were gently washed 3 times with PBS and incubated with AlexaFluor 488 Goat anti-Rabbit IgG secondary antibodies (Cell Signaling, MA, USA) (1:2000 dilution in PBS) for 2 h at room temperature (20–24°C) under dim light. Nuclei were stained with 100 μL of 2 μg/mL H33342 in PBS for 15 min at room temperature (20–24°C). Cells were washed with PBS three times before observing reactions by an IX Ultra confocal plate reader (Molecular Devices, San Diego, CA, USA), fitted with a Plan Fluor 40x NA0.6 extra-long working distance objective.

2.8 | siRNA transfection

Prior to siRNA transfection, L6 cells were seeded at 5×10^4 cells/well in 24-well plates and incubated overnight. About 16 pmol of siRNA (GRK2 siRNA [Assay ID: s129237] or negative control siRNA [Cat#4390843], Invitrogen) per well was diluted in 50 μL of Opti-MEM and 3 μL of lipofectamine RNAiMAX transfection reagents added. During 30-min incubation, medium was changed with 500 μL of Opti-MEM. The mixture was added into each well and the plates incubated for 24 h. The next day, medium was replaced with 1 mL of DMEM containing 2% FBS and further incubated for 24 h. Cells were serum-starved overnight before glucose uptake assays.

2.9 | β_2-AR internalization

CHO-GLUT4myc cells expressing SNAP-tagged β_2-ARs[26] were plated at 1×10^4 cells/well in black clear bottom 96-well microplates. The next day, 0.2 μM membrane impermeant SNAP Surface® Alexa Fluor® 488 (New England Biolabs, Ipswich, MA, USA) in the growth medium was added and incubated for 30 min at 37°C in 5% CO_2. Cells were then washed with 1× HBSS and incubated with agonists for 1 h at 37°C in 0% CO_2. Internalization was terminated by aspirating HBSS and adding 100 μL of 3% paraformaldehyde (Thermo Fisher Scientific, MA, USA) in PBS for 10 min at room temperature (20–24°C). Cells were washed with 100 μL PBS for 5 min and nuclei stained with 100 μL of 2 μg/mL Hoechst 33342 (H33342) for 15 min at room temperature (20–24°C). Cells were washed with 100 μL PBS before imaging. Plates were scanned using an IX Ultra confocal plate reader (Molecular Devices, San Diego, CA, USA), using a Plan Fluor 40× NA0.6 extra-long working distance objective.

2.10 | Real-time kinetic bioluminescence resonance energy transfer (BRET)

HEK293 cells were seeded in six-well plates at 1×10^5 cells/well and transfected with a bicistronic vector (500 ng/well) expressing the β_2-AR-Rluc8 and β-arrestin2-Venus (Figure S2) using polyethyleneimine (PEI). For GRK2 recruitment, cells were transfected with two vectors expressing the β_2-AR-Rluc8 or GRK2-Venus (100 ng/well and 400 ng/well respectively; Figure S3). The next day, cells were replated in white tissue culture-poly-D-lysine-treated 96-well microplates and grown overnight in phenol red-free DMEM with 5% FBS. About 48 h after transfection, cells were assayed in 100 μL final volume with 5 μM coelenterazine-H and ligands after serum starving for 4 h. BRET was measured at 37°C in a PHERAstar FS microplate reader (BMG LabTech). Emissions were simultaneously recorded from donor Rluc8 (475 ± 15 nm) and acceptor Venus (535 ± 15 nm). Cells were assayed before and after treatment with ligands or vehicle (serum free and phenol red-free DMEM medium). Ligand-induced BRET was calculated by subtracting the ratio of emission through the acceptor wavelength window over emission through the donor wavelength window for a vehicle-treated cell sample from the same ratio for a second aliquot of the same cells treated with ligands. The final pre-treatment reading is presented at the zero-time point (time of ligand/vehicle addition).

2.11 | RNA extraction and cDNA synthesis

RNA was extracted from L6 myoblasts and myotubes using TRIzol (Invitrogen). Extracted RNAs were assessed by NanoDrop 2000 for quality and quantity, and by electrophoresis on 1% agarose gels. Samples were stored at −80°C. About 0.5 μg of RNA was reverse-transcribed using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad). Reactions comprised 2 μL of 5× iScript reverse transcription supermix, 3 μL DNase/RNase free water and 0.5 μg of RNA in 10 μL in 200 μL Eppendorf PCR tubes. Cycles were performed on a thermal cycler (Applied Biosystems 2720) as follows: 25°C for 5 min, 42°C for 30 min, 85°C for 5 min, and cooled to 4°C. cDNA was diluted with 90 μL DNase/RNase free water to obtain the

equivalent of 20 ng/4 μL per reaction as starting RNA. The reverse transcribed samples were stored at −20°C.

2.12 | Reverse transcription PCR

PCR amplification was performed using intron-spanning primers for GRK2, GRK3, GRK4, GRK5, and GRK6 (Table S2). The reaction mix was in 20 μL; 0.5 U Platinum Pfx DNA polymerase (Invitrogen), 1× Pfx amplification buffer, 130 μmol/L dNTPs, 1.5 mmol/L $MgSO_4$, 5.8 pmol forward, and 5.8 pmol reverse primer. 1× PCR enhancer solution was added for GRK2, 3 and 4 whereas 2× PCR enhancer solution for GRK5 and 3× PCR enhancer solution for GRK6 (Invitrogen). Following initial denaturation (2 min at 95°C), each cycle consisted of 30 s at 95°C denaturation, 30 s at 60°C annealing, and 30 s at 72°C extension. A total of 28 PCR cycles were performed for GRK2/5, 30 cycles for GRK3/6, and 32 cycles for GRK4 using a MWG-Biotech Primus96 Plus. Products were electrophoresed on 1.3% agarose gels and images captured using a Typhoon TRIO imaging system (GE Healthcare, Piscataway, NJ, USA).

2.13 | Quantitative real-time polymerase chain reaction (qRT-PCR)

qPCR was performed in duplicate using TaqMan Gene Expression assays (Life Technologies) for GRK2 (*Adrbk1*; *Rn00562822_m1*) and Hrpt1 (Rn01527840_m1). Each reaction comprised 4 μL cDNA, 0.5 μL TaqMan Gene Expression Assay, 0.5 μL DNAse/RNase free water, and 5 μL TaqMan Fast Advanced Master Mix dispensed in Eppendorf twin-tec PCR plates. qPCR reactions were carried out using a CFX ConnectTM Real-Time PCR Detection System (Bio-Rad). After heating to 50°C for 2 min and denaturation at 95°C for 10 min, fluorescence was detected over 40 cycles (95°C for 15 s and 60°C for 1 min). Cq values were calculated by the Bio-Rad analysis module. Data are expressed as expression of the gene of interest relative to *Hprt1*, calculated as ($2^{-\Delta Cq}$). All statistics for gene expression were performed on ΔCq values, as these data are normally distributed. MIQE guidelines were followed. GRK2 siRNA did not affect the expression of the housekeeping gene Hprt1 (Figure S8).

2.14 | Data analysis

This was an exploratory study therefore no formal sample-size calculation was performed. Hence computed *p*-values cannot be interpreted as hypothesis-testing but only as descriptive. All data were analyzed using GraphPad Prism v9.02 (GraphPad Software, San Diego, CA, USA) using pre-programmed equations. All pooled data are represented as the mean ± standard deviation (SD) from at least 3 independent biological experiments performed in duplicate, triplicate, or quadruplicate (exception indicated). Independent experiments were individually fit and pooled parameters such as pEC_{50} and Emax were expressed as the mean ± standard deviation (SD). Bias plots and bias factors (where full concentration-response curves were generated) were analyzed as described previously.[27,28]

2.15 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY,[29] and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/24: G protein-coupled receptors.[30]

3 | RESULTS

3.1 | CHO-GLUT4myc cells expressing mutant human β_2-ARs

CHO-GLUT4myc cells stably expressing the wild-type human β_2-AR or mutant β_2-ARs (Figure S1) were generated. In the 3 mutated β_2-AR constructs, we changed the proximal and distal cassettes of the C-terminal tail (a proximal Ser/Thr cassette of the C-terminal tail—S355A, S356A, T360A and S364A and a distal Ser/Thr cassette of the C-terminal tail—T384A, T393A, S396A, S401A, S407A, T408A, and S411A) (Figure S1B), or both the proximal and distal cassettes of the β_2-AR C-terminal tail and all PKA phosphorylation sites namely S261A, S262A, S345A, and S346A (Figure S1C) or a β_2-AR mutant with just a mutated PSD95–Dlg–ZO1 (PDZ) domain binding motif—S411A (Figure S1D).

3.2 | Saturation and competition binding in CHO-GLUT4myc cells expressing the wild-type or mutant human β_2-ARs

To determine the effects of the mutations on receptor expression and binding characteristics, both saturation and competition binding experiments were performed (Figure 1). [^{3}H]-CGP12177A binding occurred in a saturable manner to a single binding site in CHO-GLUT4myc cells expressing the wild-type β_2-AR (Figure 1A; Table 1). Saturation binding in CHO-GLUT4myc cells expressing the mutant β_2-ARs also occurred in a saturable manner to a single binding site (Figure 1B–D). There was no difference in the pKd values for [^{3}H]-CGP12177A between the β_2-AR wild-type, β_2-AR (−)GRKcom, β_2-AR (−)GRK/PKA, and β_2-AR DALL (Table 1). β_2-AR DALL was expressed at comparable levels to the wild-type receptor, whereas the β_2-AR (−)GRKcom and β_2-AR (−)GRK/PKA were expressed at lower levels (Table 1). Competition binding studies were performed to determine

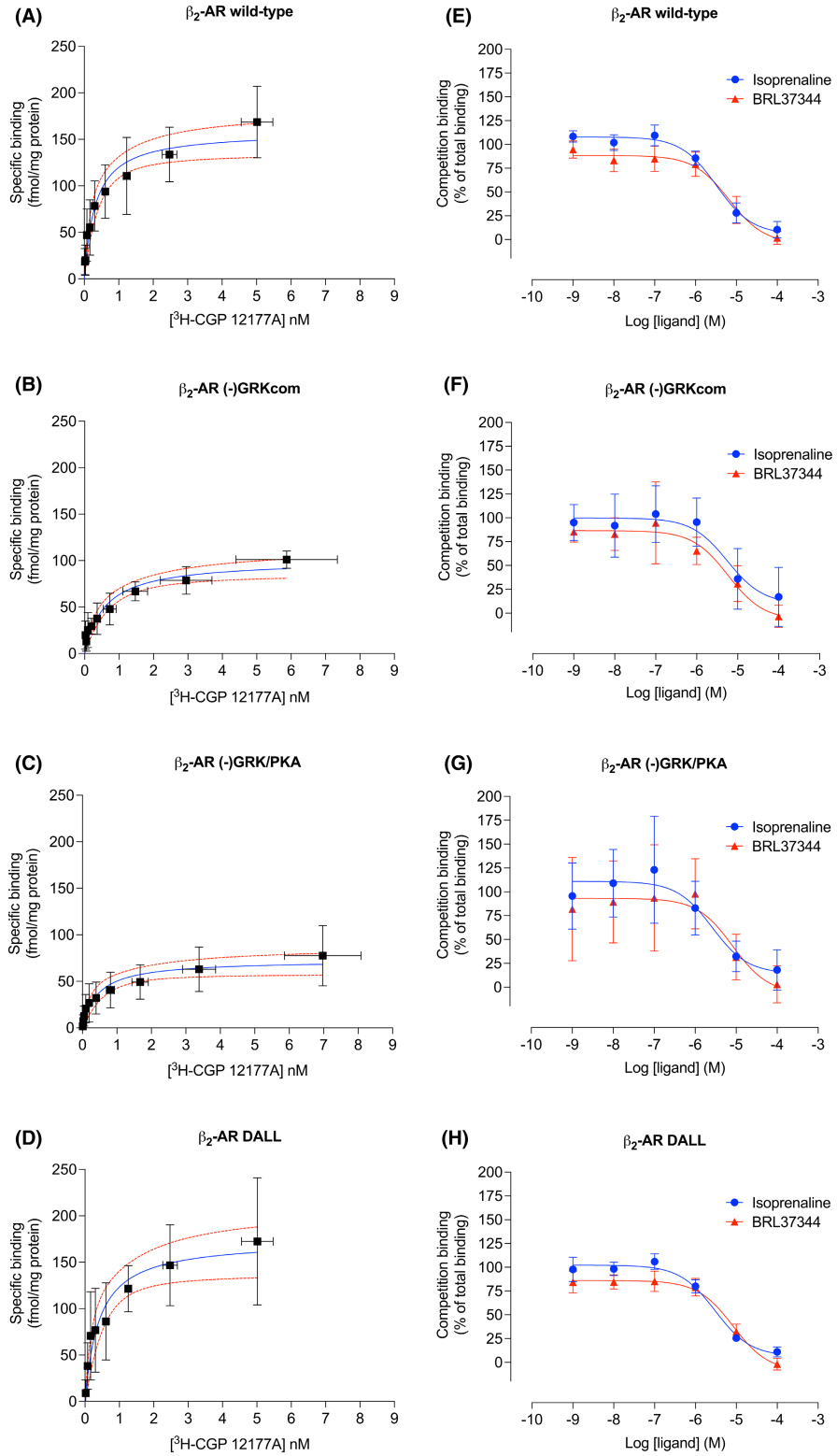

FIGURE 1 Saturation of (A–D) and competition for (E–H) [^{3}H]-CGP12177A binding in CHO-GLUT4myc cells stably expressing wild-type or mutant β_2-adrenoceptors (AR). Binding (fmol/mg protein) determined in CHO-GLUT4myc cells expressing (A) β_2-AR wild-type, (B) β_2-AR (−)GRKcom, (C) β_2-AR (−)GRK/PKA, or (D) β_2-AR DALL by incubation with [^{3}H]-CGP12177A for 2 h. Competition for [^{3}H]-CGP12177A(~2 nM) binding was determined by incubating with isoprenaline or BRL37344 for 2 h (E–H). Non-specific binding was determined with (−)-propranolol (10 μM). Points show mean ± standard deviation (SD) of 5 independent experiments performed in quadruplicate (saturation) or triplicate (competition).

the binding affinities (pKi) of isoprenaline and BRL37344 at the wild-type or mutant β_2-ARs expressed in CHO-GLUT4myc cells, and to determine whether the affinity of these ligands is affected by the mutations (Figure 1E–H). Both isoprenaline and BRL37344 affinities (pKi) were similar in wild-type, β_2-AR (−)GRKcom, β_2-AR (−)GRK/PKA, and β_2-AR DALL (Figure 1; Table 1).

3.3 | cAMP accumulation in CHO-GLUT4myc cells expressing the wild-type or mutant human β_2-ARs

In untransfected CHO-GLUT4myc cells no cAMP accumulation or glucose uptake in response to either isoprenaline or BRL37344 occurred (Figure S4), but these cells displayed a normal glucose uptake

TABLE 1 Affinity (pKd) and density of binding sites (Bmax) for [^{3}H]-CGP12177A binding and pKi values for isoprenaline and BRL37344 in CHO-GLUT4myc cells expressing wild-type or mutant β_2-ARs.

	pKd	Bmax (fmol/mg protein)	pKi	
			Isoprenaline	BRL37344
β_2-AR wild-type	9.46 ± 0.34	164.22 ± 40.24	5.48 ± 0.31	5.17 ± 0.20
β_2-AR (–)GRKcom	9.25 ± 0.39	101.97 ± 8.30*	5.21 ± 0.40	5.19 ± 0.38
β_2-AR (–)GRK/PKA	9.28 ± 0.42	74.49 ± 22.16**	5.59 ± 0.20	5.07 ± 0.24
β_2-AR DALL	9.35 ± 0.36	171.60 ± 48.29	5.53 ± 0.13	5.07 ± 0.18

Note: pKd and B_{max} values were calculated from saturation binding experiments (Figure 1A–D) or competition binding curves (Figure 1E–H). Values represent mean ± standard deviation (SD) of 5 independent experiments performed in triplicate or quadruplicate. *$p < .05$, **$p < .01$ compared to the β_2-AR wild-type (one-way ANOVA Dunnett's multiple comparisons test).

response to insulin that was maintained after transfection with β_2-AR variants (Figure S5; Table S1). cAMP accumulation increased in a concentration-dependent manner with isoprenaline or BRL37344 in CHO-GLUT4myc cells expressing the wild-type human β_2-AR (Figure 2A), and this response was retained in cells expressing mutant human β_2-ARs (Figure 2B–D). There was little difference between pEC_{50} and Rmax values obtained for β_2-AR variants, but there was a difference ($p < .05$ for cAMP for BRL37344) in Rmax value of β_2-AR DALL compared to β_2-AR wild-type (Table 2). These differences in Rmax values were normalized to the reference isoprenaline (10 μM) response. We previously showed that BRL37344 is a partial agonist for cAMP accumulation in L6 myotubes[5] and it behaved similarly here (Figure 2). Thus, BRL37344 displayed low efficacy and low potency ($pEC_{50} < 6$) for cAMP generation whereas isoprenaline acted as a full agonist with approximately 25-fold greater potency. The responses to the maximal BRL37344 concentration tested (10 μM) varied between 28.4% and 48.8% of the response to isoprenaline (10 μM).

3.4 | [^{3}H]-2-deoxyglucose uptake and GLUT4 translocation in CHO-GLUT4myc cells expressing the wild-type or mutant human β_2-ARs

Both isoprenaline and BRL37344 increased glucose uptake (Figure 2E–H) and GLUT4 translocation (Figure 2I–L) in CHO-GLUT4myc cells expressing β_2-AR or β_2-AR variants. For [^{3}H]-2-deoxyglucose uptake, pEC_{50} values for isoprenaline were similar to those obtained for cAMP accumulation although, in contrast to cAMP responses, maximum responses within the group appeared more closely related to receptor expression (Table 2; Figure 2). For GLUT4 translocation (Figure 2I–L), isoprenaline was about 5–6 times more potent compared to [^{3}H]-2-deoxyglucose uptake (Figure 2E–H; Table 2). BRL37344 also displayed similar pEC_{50} values and maximal responses across all β_2-AR variants (Figure 2E–L; Table 2). However, and in marked contrast to isoprenaline, BRL37344 was 44 times more potent for promotion of [^{3}H]-2-deoxyglucose uptake (Figure 2E–H) than for cAMP accumulation (Figure 2A–D) and was a high efficacy partial agonist or a full agonist for glucose uptake (Table 2). Likewise, for GLUT4 translocation (Figure 2I–L) BRL37344 was 110 times more potent than for cAMP accumulation and behaved as a high efficacy agonist (Table 2). Thus, although BRL37344 was a poor partial agonist for cAMP generation it displayed similar potency and efficacy to isoprenaline for glucose uptake and GLUT4 translocation. These studies demonstrate that large changes in glucose uptake can be produced by compounds that have only weak ability to promote cAMP accumulation. Qualitative differences in response between [^{3}H]-2-deoxyglucose uptake and GLUT4 translocation may be explained by the differences in experimental conditions utilized in these assays.

3.5 | Biased agonism of BRL37344 at the wild-type or mutant human β_2-ARs

Bias plots for isoprenaline and BRL37344 at the wild-type or mutant human β_2-AR were generated (Figure S6). Isoprenaline appears to preferentially increase GLUT4 translocation relative to cAMP generation at all 4 β_2-ARs, and trends away from glucose uptake at the DALL and GRK/PKA β_2-AR mutants relative to cAMP generation. Isoprenaline also preferences GLUT4 translocation relative to glucose uptake only at the DALL β_2-AR mutant. Since cAMP accumulation in response to BRL37344 did not reach a maximal response, quantitative comparisons cannot be fully made against this signaling output. BRL37344 appears biased toward glucose uptake and GLUT4 translocation versus cAMP generation at all 4 β_2-ARs (Figure S7).

3.6 | β_2-AR-mediated receptor internalization in CHO-GLUT4myc cells

Receptor internalization following isoprenaline or BRL37344 was performed in cell lines expressing SNAP-tagged receptor variants. β_2-ARs were at the cell surface in unstimulated CHO-GLUT4myc cells stably expressing either wild-type or mutant β_2-AR containing an N-terminal SNAP tag. Isoprenaline caused translocation of the SNAP-tagged β_2-AR wild-type (Figure 3A) or the corresponding β_2-AR DALL mutant lacking a PDZ motif (Figure 3D) from the cell surface to intracellular compartments within 30 min. However, internalization to isoprenaline was not observed with SNAP-tagged receptors lacking either the C-terminal GRK phosphorylation sites (β_2-AR (–)GRKcom) or both GRK/PKA phosphorylation sites (β_2-AR (–)GRK/PKA) (Figure 3B,C). BRL37344 failed to cause internalization of any of the SNAP-tagged β_2-AR

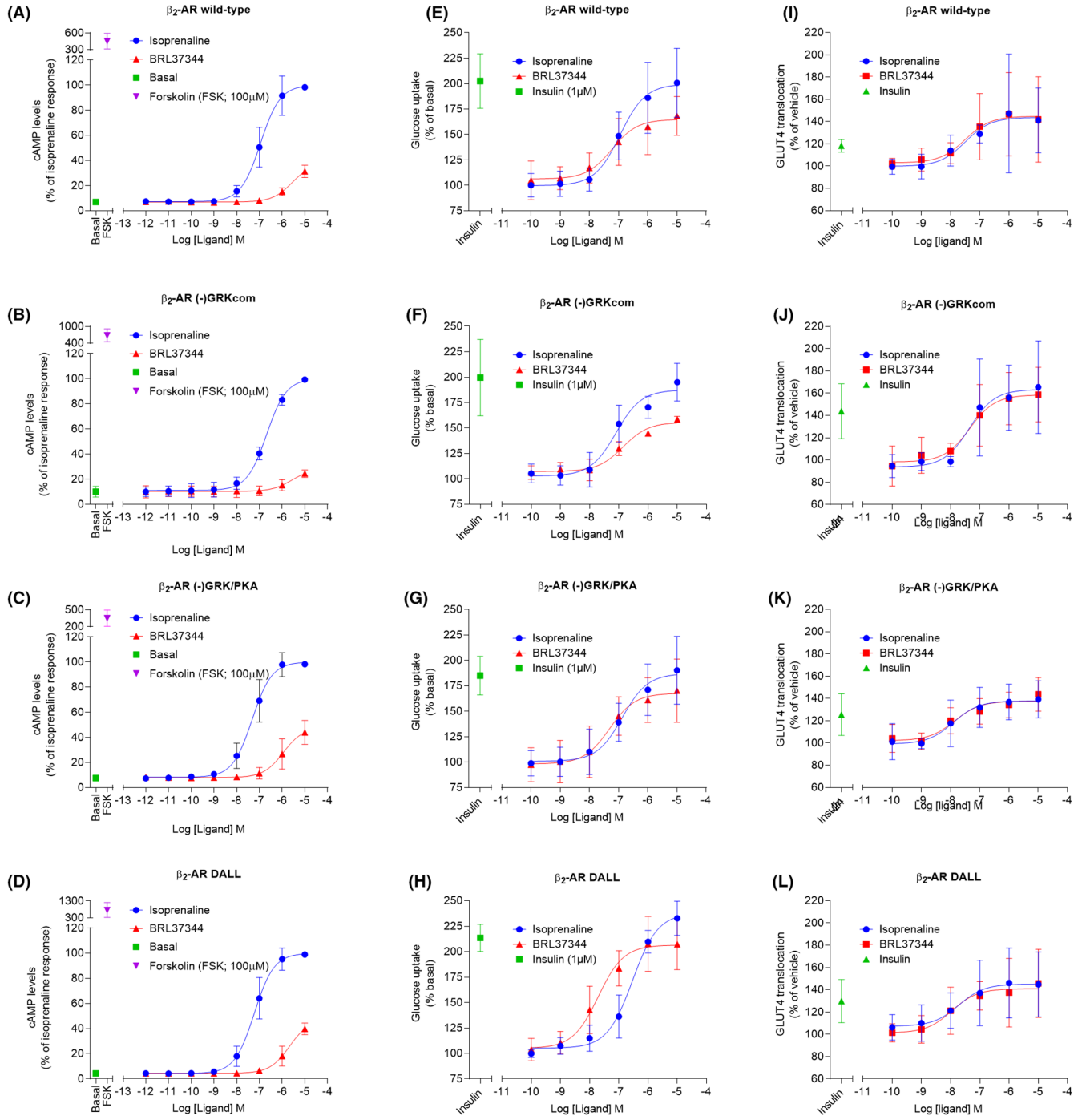

FIGURE 2 Concentration-dependent cAMP accumulation (A–D), glucose uptake (E–H), and GLUT4 translocation (I–L) in response to isoprenaline or BRL37344 in CHO-GLUT4myc cells stably expressing β_2-adrenoceptor (AR) variants. cAMP was measured in CHO-GLUT4myc cells expressing either (A) β_2-AR wild-type, (B) β_2-AR (–)GRKcom, (C) β_2-AR (–)GRK/PKA, or (D) β_2-AR DALL treated with isoprenaline or BRL37344 for 30 min in the presence of 0.5 mM IBMX. Results are expressed as % of cAMP produced by isoprenaline (10 μM). 2-deoxy-[^{3}H]-glucose uptake was measured in (E) β_2-AR wild-type, (F) β_2-AR (–)GRKcom, (G) β_2-AR (–)GRK/PKA, or (H) β_2-AR DALL following stimulation with isoprenaline, BRL37344 or insulin (10 μM) for 2 h. GLUT4 translocation was measured after 2 h stimulation with isoprenaline, BRL37344, or insulin (1 μM) in (I) β_2-AR wild-type, (J) β_2-AR (–)GRKcom, (K) β_2-AR (–)GRK/PKA, or (L) β_2-AR DALL. Data was quantified by automated multiwave scoring. Values are mean ± standard deviation (SD) of 5–6 independent experiments performed in duplicate. Data are normalized to values in vehicle-treated cells at 2 h.

variants (Figure 3A–D). The complete absence of internalization to isoprenaline in cells expressing β_2-AR (–)GRKcom or β_2-AR (–)GRK/PKA confirms that receptor phosphorylation by GRK following exposure to a full agonist is a prerequisite for receptor internalization.[7,11] The weak partial agonist for cAMP accumulation, BRL37344 did not promote GRK mediated receptor phosphorylation or internalization.

TABLE 2 pEC_{50} values and maximal responses to isoprenaline and BRL37344 for cAMP accumulation, [^{3}H]-2-deoxyglucose uptake and GLUT4 translocation in CHO-GLUT4myc cells expressing β_2-AR variants.

	cAMP accumulation		[^{3}H]-2-deoxyglucose uptake		GLUT4 translocation	
	pEC50	Response at 10 μM ISO (%)	pEC50	R_{max} (% of basal)	pEC50	R_{max} (% of basal)
Isoprenaline						
β_2-AR wild-type	6.91 ± 0.38	100.00	6.94 ± 0.11	199.72 ± 35.04	7.60 ± 0.42	143.34 ± 40.90
β_2-AR (−)GRKcom	6.68 ± 0.08	100.00	7.12 ± 0.25	187.40 ± 12.00	9.12 ± 3.47	156.86 ± 41.75
β_2-AR (−)GRK/PKA	7.30 ± 0.29	100.00	7.01 ± 0.63	187.08 ± 32.21	7.57 ± 0.89	141.34 ± 18.85
β_2-AR DALL	7.22 ± 0.29	100.00	6.64 ± 0.40	237.10 ± 21.48	7.95 ± 0.63	145.44 ± 31.65
BRL37344						
β_2-AR wild-type	5.49 ± 0.28	39.10 ± 2.75	7.16 ± 0.21	165.67 ± 21.55	7.61 ± 0.19	143.34 ± 37.20
β_2-AR (−)GRKcom	5.56 ± 0.20	28.69 ± 2.73	6.89 ± 0.38	156.00 ± 4.38	7.73 ± 1.03	157.34 ± 28.51
β_2-AR (−)GRK/PKA	5.88 ± 0.40	49.32 ± 9.19	7.42 ± 0.33	167.44 ± 27.71	7.68 ± 0.65	140.16 ± 13.72
β_2-AR DALL	5.57 ± 0.50	54.73 ± 16.06*	7.75 ± 0.38*	206.70 ± 24.01*	7.95 ± 0.09	141.12 ± 26.55

Note: pEC_{50} and R_{max} values were calculated from concentration-response curves for cAMP accumulation (Figure 2A–D) after 30 min agonist exposure in the presence of 0.5 mM IBMX; [^{3}H]-2-deoxyglucose uptake measured for 15 min after 150 min agonist exposure (Figure 2E–H); and GLUT4 translocation after 120 min agonist exposure (Figure 2I–L). Values represent mean ± standard deviation (SD) from 5 to 6 experiments performed in duplicate. No pEC_{50} or Rmax values were different from the β_2-AR wild-type in the isoprenaline group whereas Rmax values of both cAMP accumulation and glucose uptake and pEC_{50} of glucose uptake were different compared to the β_2-AR wild-type in the BRL37344 group (one-way ANOVA Dunnett's multiple comparisons test; $^*p < .05$).

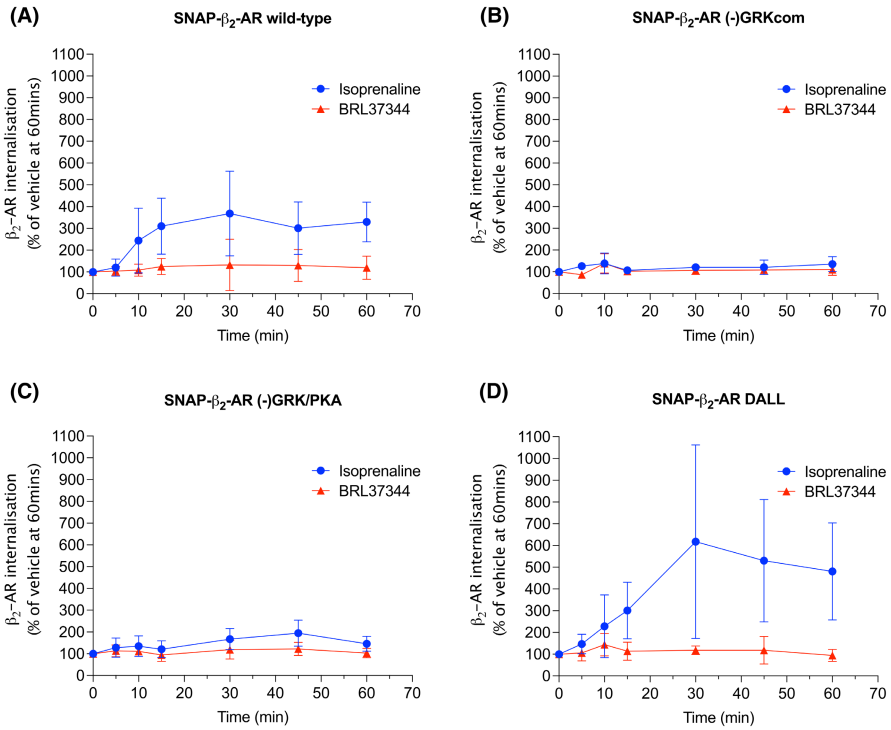

FIGURE 3 Time course of SNAP-β_2-AR internalization in response to isoprenaline or BRL37344. Time course of β_2-AR internalization following stimulation with 10 μM isoprenaline or 10 μM BRL37344 in (A) SNAP-β_2-AR wild-type, (B) SNAP-β_2-AR (−)GRKcom, (C) SNAP-β_2-AR (−)GRK/PKA, or (D) SNAP-β_2-AR DALL cells. Data were quantified using an automated granularity algorithm. Values are mean ± standard deviation (SD) in 5 independent experiments performed in duplicate. Data are normalized to values in vehicle-treated cells at 60 min.

3.7 | Isoprenaline, but not BRL37344, recruits β-arrestin2 to the phosphorylated receptor

Receptors phosphorylated by GRKs bind β-arrestins, leading to internalization and desensitization. We directly monitored the interactions between the β_2-AR variants and β-arrestin2 using a BRET assay. For the BRET assay, bicistronic vectors that have β-arrestin2-Venus with either the β_2-AR wild-type-hRluc8, β_2-AR (−)GRKcom-hRluc8, β_2-AR (−)GRK/PKA-hRluc8, or β_2-AR DALL-hRluc8 were generated (Figure S2). Isoprenaline (1 μM), but not BRL37344 (1 μM), caused an increase in the BRET signal between β_2-AR wild-type-hRluc8 (Figure 4A–C) or β_2-AR DALL-hRluc8 (Figure 4C) and β-arrestin2-Venus. However, recruitment of β-arrestin2 following isoprenaline (1 μM) was markedly reduced in both β_2-AR (−)GRKcom-hRluc8 (Figure 4A) and β_2-AR (−)GRK/PKA-hRluc8 (Figure 4B) and absent for all β_2-AR variants treated with BRL37344 (1 μM). Again, this strongly

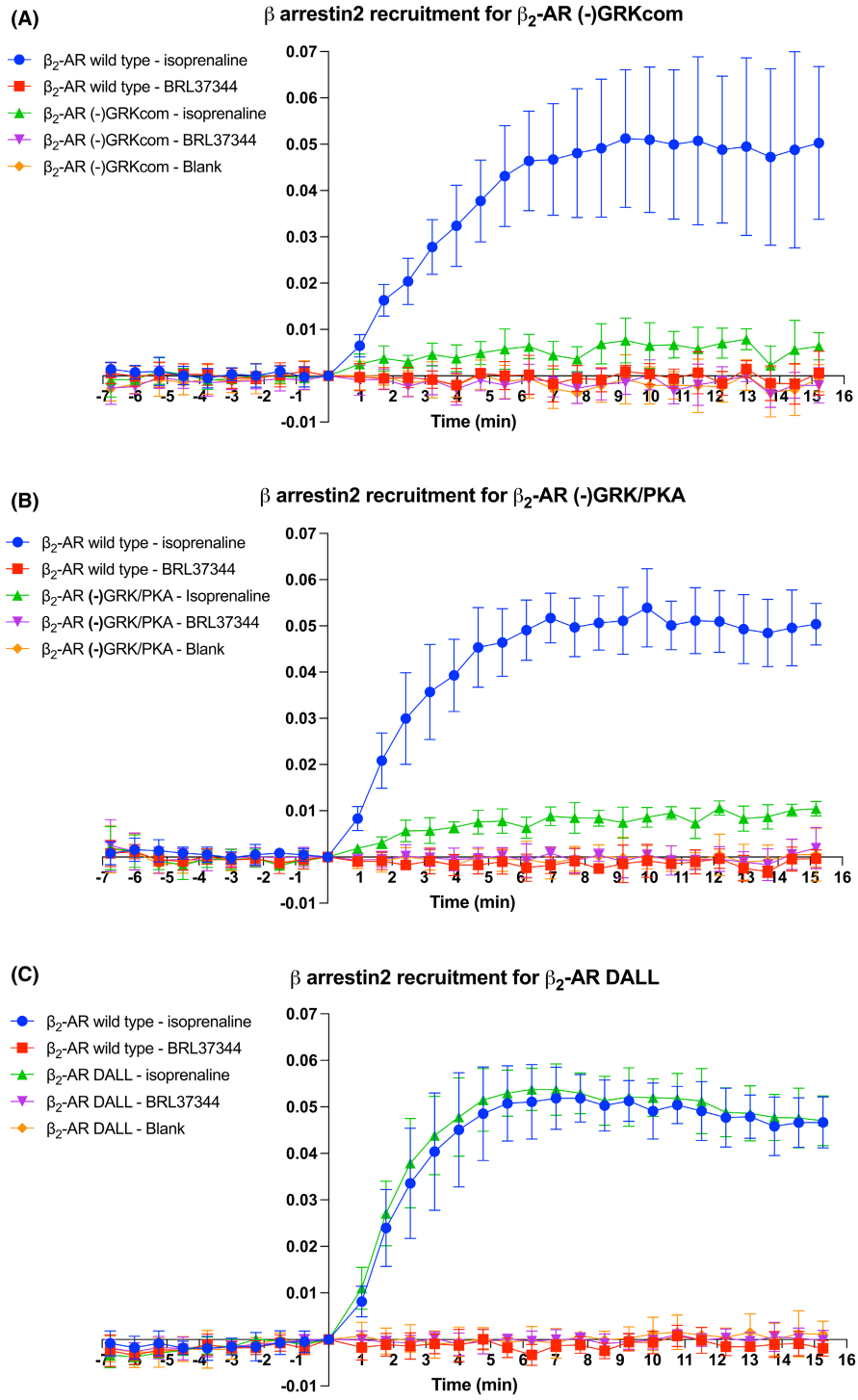

FIGURE 4 Interaction between the β_2-adrenoceptor and β-arrestin2 in HEK293 cells examined by bioluminescent resonance energy transfer (BRET). HEK293A cells were transfected with bicistronic vectors encoding β-arrestin2-venus with Rluc8 tagged β_2-AR wild-type compared to (A) β_2-AR (−)GRKcom, (B) β_2-AR (−)GRK/PKA, or (C) β_2-AR DALL. About 48 h after transfection, cells were assayed in a 100 μL final volume with 5 μM coelenterazine-H and either isoprenaline or BRL37344 (1 μM) after serum starving for 4 h. Results are mean ± standard deviation (SD), $n = 5$.

suggests that strong agonism (high efficacy) and GRK phosphorylation of the receptor is a prerequisite for recruitment of β-arrestin2.

3.8 | Effects of GRK2 inhibition on β-arrestin2 recruitment and [^{3}H]-2-deoxyglucose uptake in rat L6 skeletal muscle cells

We have previously shown that both isoprenaline and BRL37344 stimulate glucose uptake in rat skeletal muscle L6 cells (myotubes) that endogenously express the β_2-AR.[5] Figure 5A shows that undifferentiated L6 myoblasts and differentiated L6 myotubes express GRK2 = GRK6 > GRK5 > GRK4. Transfection of L6 myoblasts with either control (scrambled) siRNA or GRK2 siRNA was conducted 72 h before experiment. Cells transfected with control (scrambled) siRNA showed no change of GRK2 from control whereas cells transfected with GRK2 siRNA showed knock-down of GRK2 mRNA (Figure 5B). To further study the role of GRK2 in isoprenaline or BRL37344-stimulated glucose uptake, the effects of GRK inhibitors and siRNA knockdown of GRK on β-arrestin2 recruitment and

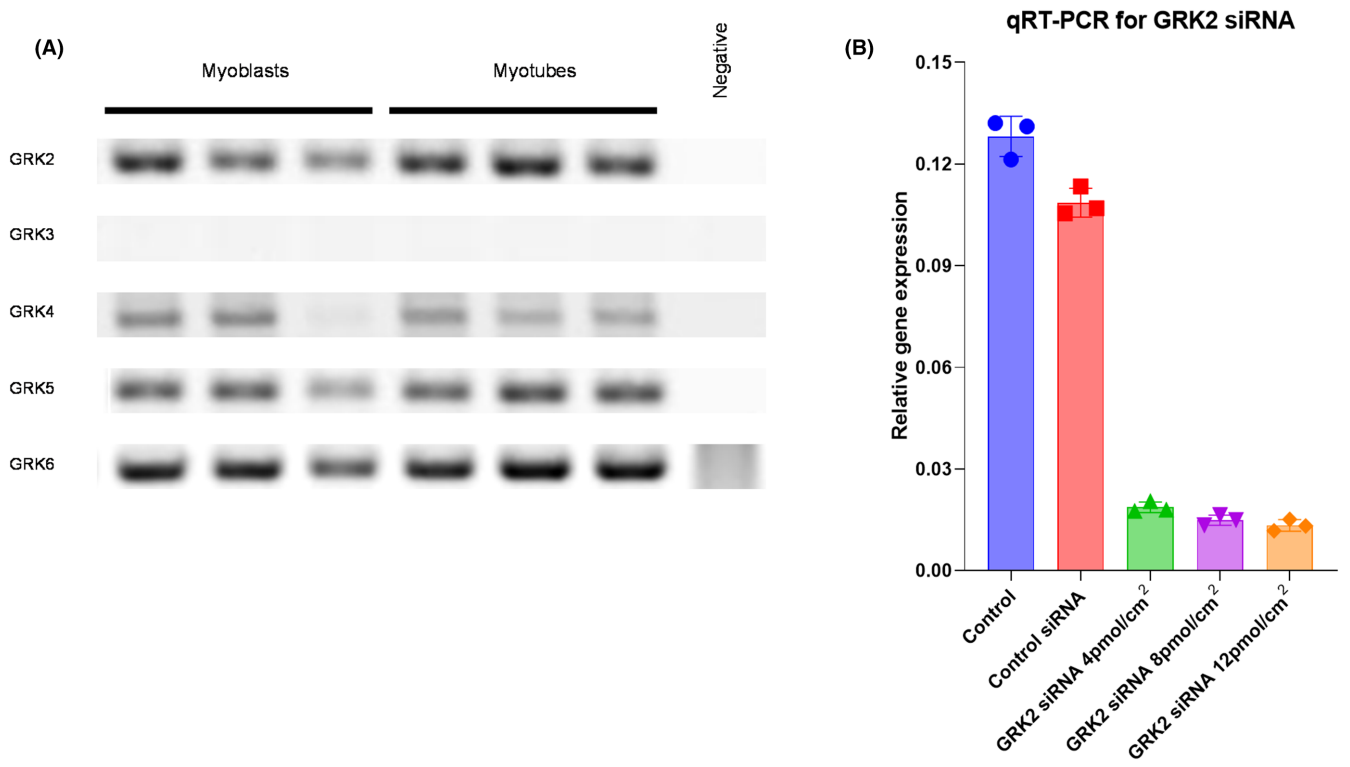

FIGURE 5 GRK isoform mRNA expression in L6 cells and effect of siRNA knockdown on GRK2 mRNA expression. (A) RT-PCR of GRK isoforms in L6 cells in 3 independent experiments. (B) Knockdown of GRK2 mRNA following transfection of GRK2 siRNA. Relative GRK2 gene expression was determined by qRT-PCR. Results are mean ± standard deviation (SD), $n = 3$.

[^{3}H]-2-deoxyglucose uptake were examined. BRET studies of interactions between β_2-AR-Rluc8 and β-arrestin2-venus (Figure 6A) showed that isoprenaline (100 nM) treatment caused a maximal BRET signal within 10 min. Pre-treatment with the GRK inhibitors paroxetine or CMPD101 (both 10 μM) reduced the BRET signal between the receptor and β-arrestin2 (Figure 6A) suggesting GRK phosphorylation was required for this interaction. The β_2-AR partial agonist BRL37344 (100 nM) did not increase the β_2-AR/β-arrestin BRET signal at any time point (Figure 6A). GRK2 inhibition by paroxetine and CMPD101 (both 10 μM) was also associated with a decrease in glucose uptake in L6 cells (Figure 6B–D). However, the most effective reduction in glucose uptake was produced in cells in which GRK2 had been knocked down by siRNA pre-treatment where there was a marked reduction of isoprenaline-stimulated glucose uptake that was not observed following transfection of the scrambled control siRNA (Figure 6F).

3.9 | Direct interaction between β_2-AR variants and GRK2

Since isoprenaline caused both GLUT4 translocation and increased glucose uptake in cells expressing variants of the β_2-AR lacking phosphorylation sites targeted by GRK2 we examined whether there was an interaction between β_2-AR variants and GRK2 independent of phosphorylation. HEK293A cells were transfected with GRK2-venus and either Rluc8 tagged wild type β_2-AR, β_2-AR (–)GRKcom, β_2-AR (–)GRK/PKA, or β_2-AR DALL. About 48 h after transfection the interaction between receptors and GRK2 was examined by BRET. Isoprenaline (1 μM) addition caused a BRET signal not only in cells expressing wild type β_2-AR and β_2-AR DALL receptors but also in cells expressing β_2-AR (–)GRKcom and β_2-AR (–)GRK/PKA that do not undergo GRK-mediated phosphorylation (Figure 7). Cells expressing receptors with intact GRK phosphorylation sites produced a larger signal, but the smaller signal associated with receptors that do not undergo phosphorylation suggests that GRK2 can associate with β_2-AR independently of phosphorylation and that this interaction may underpin its function as a scaffold protein.

4 | DISCUSSION

There is evidence that β_2-AR mediated glucose uptake occurs through both cAMP-dependent and cAMP-independent mechanisms, since (i) glucose uptake occurs in response to membrane-permeable cAMP analogs such as 8Br-cAMP[3] yet (ii) β_2-AR mediated glucose uptake is only partially blocked by inhibition of PKA.[2–4] In addition, some β_2-AR partial agonists such as BRL37344, that produce only modest amounts of cAMP, increase glucose uptake and GLUT4 translocation with similar potency and efficacy to isoprenaline.[5] There is also evidence suggesting that, in addition to phosphorylation of agonist-occupied receptors, GRK2 is involved in glucose

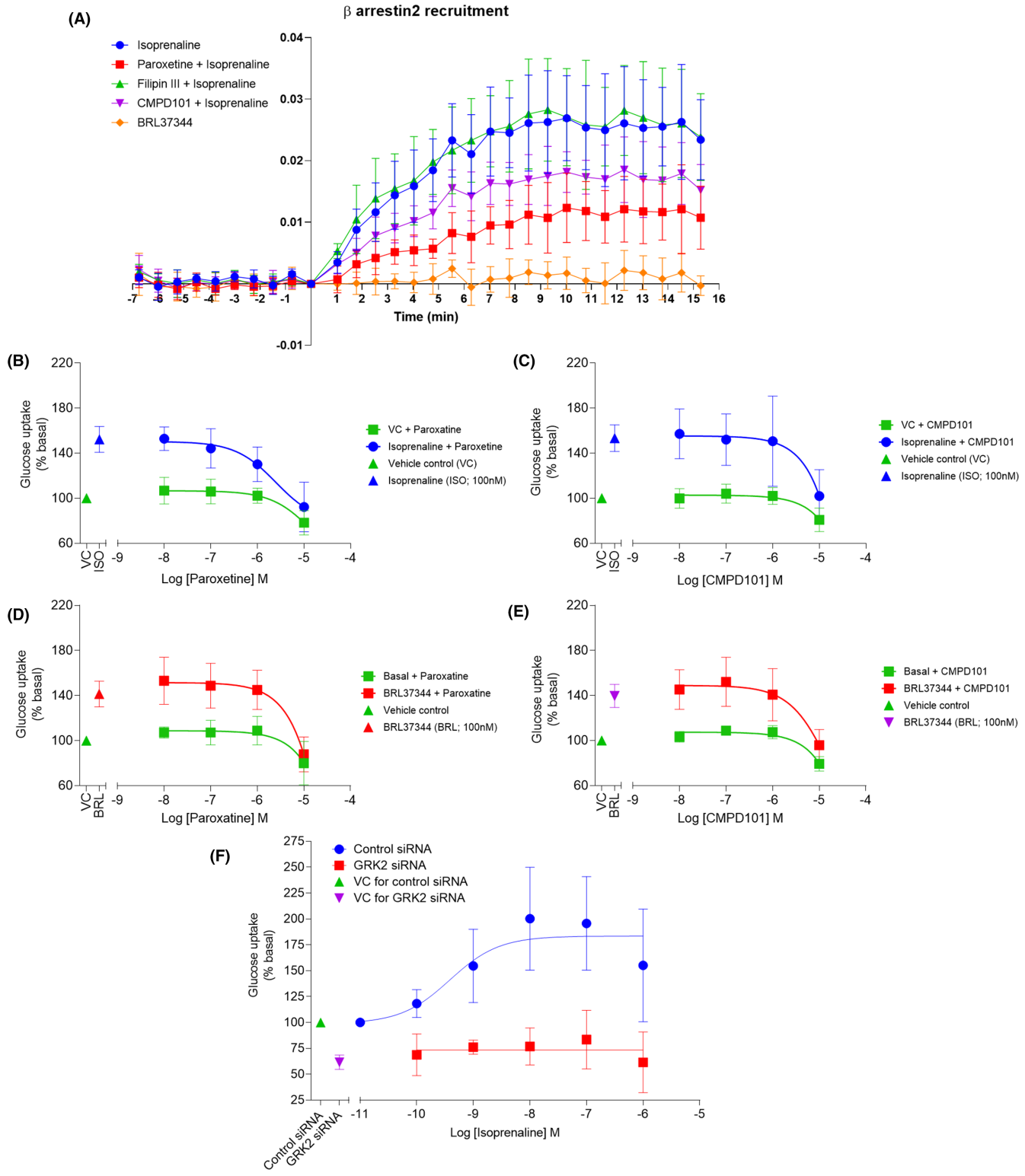

FIGURE 6 Effects of GRK2 inhibition on β-arrestin2 recruitment in HEK cells and glucose uptake in rat L6 skeletal muscle cells. (A) BRET assay was performed to investigate the effect of GRK2 inhibition on β-arrestin2 recruitment using a bicistronic vector expressing the β_2-AR and β-arrestin2 in HEK293A cells. The GRK inhibitors paroxetine and CMPD101 (both 10 μM) reduced BRET between the β_2-AR and β-arrestin2 following isoprenaline (100 nM). BRL37344 (100 nM) did not cause β-arrestin2 recruitment. In L6 cells, paroxetine and CMPD101 (10 μM) also reduced glucose uptake in response to isoprenaline (100 nM; B,C) and BRL37344 (10 μM; D,E). Concentration-dependent increases in glucose uptake to isoprenaline (2 h) were observed in L6 myoblasts transfected with siRNA (GRK2 scrambled) but completely inhibited in myoblasts following transfection with GRK2 siRNA (F). Data are normalized to values from vehicle-treated cells at 2 h. All data are mean ± standard deviation (SD) in 5 independent experiments performed in duplicate.

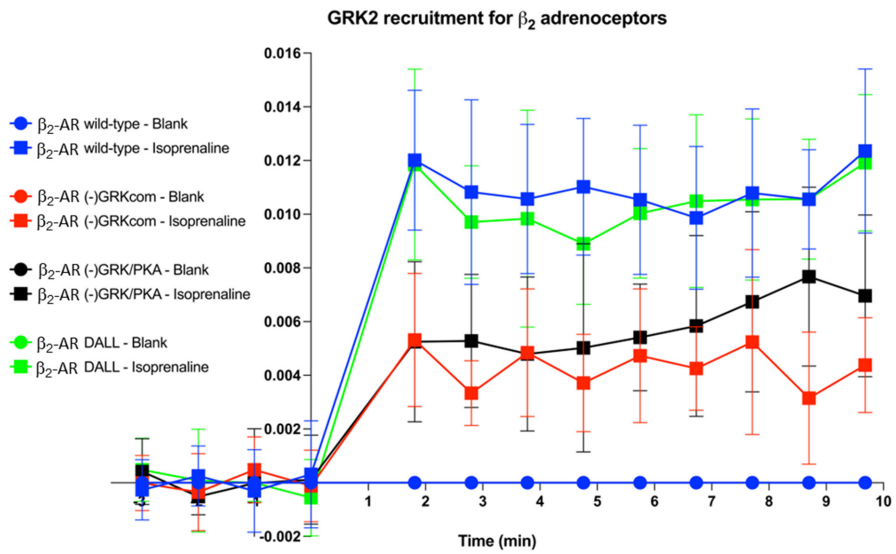

FIGURE 7 Interaction between the β_2-adrenoceptor and GRK2 in HEK293 cells examined by bioluminescent resonance energy transfer (BRET). HEK293A cells were transfected with vectors encoding GRK2-venus and either Rluc8 tagged β_2-AR wild-type, β_2-AR (−)GRKcom, β_2-AR (−)GRK/PKA, or β_2-AR DALL. About 48 h after transfection, cells were assayed in a 100 μL final volume with 5 μM coelenterazine-H and ligands [1 μM for isoprenaline] after serum starving for 4 h. Results are mean ± standard deviation (SD), $n = 9$.

metabolism[31–34] and we showed previously that isoprenaline treatment of cells overexpressing a kinase-dead GRK2 (K220R) mutant fails to increase GLUT4 translocation.[22] Hence, this study aimed to further examine the role of GRK in β_2-AR-mediated glucose uptake and in particular if GRK-mediated phosphorylation of the β_2-AR C-terminal tail is involved in the metabolic response to isoprenaline and BRL37344.

We used CHO-GLUT4myc cells stably expressing β_2-AR wild-type or three β_2-AR constructs in which serine and threonine residues comprising potential GRK/PKA phosphorylation sites were mutated to alanine (Figure S1). Clonal cell lines expressing mutant receptors were evaluated for receptor expression that was somewhat higher than reported for β_2-ARs in crude membrane preparations from human rectus abdominis muscle.[35] Clones with relatively low receptor expression were chosen to avoid promiscuous coupling to other signaling pathways.[36] Similar to previous studies,[5] in CHO-GLUT4 cells expressing the β_2-AR wild-type, BRL37344 stimulated GLUT4 translocation and glucose uptake to a similar extent to isoprenaline, with only a minor increase in cAMP production (Figure 2). Isoprenaline, but not BRL37344, also caused receptor internalization in a time-dependent manner in CHO-GLUT4myc cells stably expressing SNAP tagged β_2-AR wild-type, increased interaction between β-arrestin2 and β_2-AR-hRluc8 in BRET studies and caused functional desensitization of cAMP responses. Taken together, this showed that BRL37344 increases glucose uptake and GLUT4 translocation with a potency and efficacy comparable to that displayed by isoprenaline yet has low potency and efficacy for cAMP production and does not cause receptor interaction with β-arrestins, desensitization, or internalization. The high potency and efficacy of compounds such as BRL37344 for glucose uptake into skeletal muscle combined with low potency and efficacy for cAMP generation in other tissues suggests that compounds with similar properties could have therapeutic potential for the treatment of type II diabetes.[4,5,37] Improved glucose disposal in response to β_2-AR agonists has also been reported in humans[38–40] although the agonists used, terbutaline or clenbuterol, are high efficacy partial agonists increasing the potential for unwanted side effects (see below). As previously suggested,[5] our results show that the increase in cAMP produced by isoprenaline or BRL37344 promotes GLUT4 translocation that then remains at the cell surface for a prolonged period[41] and is available to facilitate glucose uptake. Thus, while there may be a difference in the rate at which GLUT4 translocation occurs in response to isoprenaline or BRL37344, at steady state the rate of glucose uptake is similar.[5] The disadvantage of a full agonist such as isoprenaline or high efficacy partial agonists is that the large and rapid increase in cAMP they produce is potentially associated with a plethora of side effects and desensitization whereas with BRL37344 the increase in cAMP is much smaller and not associated with desensitization[5] or associated unwanted effects that occur particularly in cardiovascular tissues.

GLUT4 translocation to the cell surface in response to isoprenaline and BRL37344 was performed in CHO-GLUT4myc cells expressing β_2-AR variants. The human GLUT4 carries a c-myc epitope within the first extracellular loop[23] that is only recognized by the primary antibody when at the cell surface. Treatment with isoprenaline or BRL37344 increased GLUT4myc translocation to the cell surface in a concentration-dependent manner not only in CHO-GLUT4myc cells expressing the β_2-AR wild-type or the β_2-AR lacking a phosphorylation site in the terminal PDZ domain (Figure 2I–L) but also in β_2-ARs lacking phosphorylation sites. For this response, there was no difference in the potency of isoprenaline or BRL37344 for any of the variants compared to the wild-type receptor (Figure 2I–L; Table 2). However, the maximum response in cAMP accumulation for GRKcom, GRK/PKA, and DALL was different from wild type whereas maximum response for glucose uptake was only different in DALL (Table 2). The positive control, insulin, increased GLUT4 translocation in all cell lines examined, (Figure S3; Table S1) showing that they remained viable.

The findings with GLUT4 translocation were reflected in the studies of glucose uptake that were also similar in cells expressing any of the β_2-AR variants. BRL37344 displayed similar

potency to isoprenaline but tended to have lower efficacy. This strongly indicated that β_2-AR-mediated glucose uptake and GLUT4 translocation occur independently of GRK-mediated receptor phosphorylation. However, SNAP-tagged receptors lacking GRK or GRK/PKA phosphorylation sites did show the clear expected phenotype with isoprenaline-stimulated β_2-AR internalization being completely abolished although GLUT4 translocation was retained to both isoprenaline and BRL37344. While the role of GRK phosphorylation in internalization[42,43] was lost in β_2-AR (−)GRKcom, and β_2-AR (−)GRK/PKA these mutants still increased cAMP production (Figure 2) that could explain the retention of GLUT4 translocation and glucose uptake.[4] However, although GRK *phosphorylation* of the β_2-AR did not influence receptor functions other than internalization, our original study still indicated a role for GRK in glucose uptake[22] since, truncation of the β_2-AR at position 349 to remove GRK phosphorylation sites or position 344 to remove GRK and PKA phosphorylation sites, did not affect cAMP accumulation but decreased glucose uptake in response to β_2-AR agonists.[22] Inhibition of GRKs by co-transfection with the pleckstrin homology domain of the C-terminus of GRK2 termed βARKct that sequesters Gβγ subunits and prevents kinase recruitment to activated receptors[42,44] or by overexpression of a kinase-dead mutant (K220R) of GRK2 also inhibited glucose uptake and GLUT4 translocation.[22] Thus, while recruitment of GRKs to the receptor is important for GLUT4 translocation and glucose uptake with the C-terminus of the receptor being necessary for this process, phosphorylation of the receptor is not, suggesting that the important property of GRKs here is as scaffold proteins.[45]

In HEK293 cells expressing the β_2-AR, the cAMP response following isoprenaline stimulation was promoted by GRK6 but not GRK2, GRK3, or GRK5 silencing.[46] More recently, it has been shown that GRK2, 3, 5, and 6 can all phosphorylate the β_2-AR with the relative importance of the variants in β-arrestin recruitment largely due to cell location and level of expression. In HEK293 cells, GRK2 and 6 were the most highly expressed with GRK2 predominating in the cytosol and GRK6 in the cell membrane.[13] In rat aortic smooth muscle cells, isoprenaline increased β_2-AR mediated cAMP accumulation following siRNA-depletion of GRK2 or GRK5 but not GRK6 and double depletion of GRK2 and GRK5 further enhanced cAMP accumulation.[47] The apparent anomaly may arise from the comparison of the removal of GRK phosphorylation sites with the removal of GRKs and as seen in the current studies these do not always equate since glucose uptake is preserved in cells expressing receptors with phosphorylation sites removed. It is also possible that the pattern of cAMP response mediated by wild-type and some of the mutant β_2-ARs arises from altered spatiotemporal kinetic profiles at the plasma membrane and within endosomes, as reported for other Gαs-coupled receptors.[48] In turn, observation of endosomal signaling may depend on the cell type examined, and also on receptor abundance. More recently, autoinhibitory roles of the β_2-AR ICL3 and C terminus have also been recognized that regulate G protein activation without the participation of arrestins. These studies suggest that the C terminus remains disordered and forms multiple weak and transient interactions with the cytoplasmic surface that are reduced following binding of agonists to the receptor.[49] This may facilitate interaction of the receptor with signaling and scaffold proteins.[49] How these direct interactions are influenced by GRK recruitment or phosphorylation is yet to be clarified.

This could suggest that GRKs influence glucose uptake by kinase activity that targets proteins downstream of the β_2-AR, or by acting as scaffold proteins. GRK2 knock-down using GRK2 siRNA or GRK inhibitors reduced glucose uptake to β_2-AR stimulation in L6 cells. GRKs are known to interact directly with various signaling proteins, and modulate downstream signaling cascades.[14] Binding partners include PI3K,[20] the PI3K downstream target, Akt,[21] insulin receptor substrate 1[50] and ERK/2—mitogen-activated protein kinase kinase (MEK).[51] Studies of β_2-AR mediated glucose uptake to isoprenaline in L6 myotubes suggest that Akt is not involved,[4] and inhibition of MEK1(upstream of Erk1/2) does not reduce BRL37344-stimulated glucose uptake, suggesting that ERK1/2 and MEK are also not important.[20] Other GRK binding partners such as clathrin[52] and caveolin,[53] that mediate internalization of β-AR, do not appear to be involved as glucose uptake was independent of desensitization.

Another explanation of the differing effects of isoprenaline and BRL37344 may arise from biased agonism: the ability of ligands to differentially activate different receptor signaling responses. Biased agonism occurs at β-ARs, which has been reviewed previously.[36,54] While bias can be quantified,[27,28] a limitation of quantifying bias for BRL37344 in this study includes the incomplete cAMP accumulation curves generated, and the different time points used (30 min for cAMP accumulation, 2 h for GLUT4 translocation and glucose uptake), with kinetics an important factor in biased agonism.[55] Our results indicate that BRL37344 may be biased away from cAMP toward glucose uptake and GLUT4 translocation at all 4 β_2-AR receptors, but further investigation into any biased agonism will need to be investigated in future studies using a wider range of signaling outputs.

5 | CONCLUSION

Our findings show that GRK phosphorylation of the β_2-AR is not required for isoprenaline or BRL37344-stimulated glucose uptake. In β_2-AR wild-type, β_2-AR (−)GRKcom, β_2-AR (−)GRK/PKA, and β_2-AR DALL both β-AR agonists caused GLUT4 translocation and glucose uptake with similar potency and efficacy. In β_2-ARs without GRK phosphorylation sites, isoprenaline increased glucose uptake but failed to promote receptor internalization. BRL37344 stimulates glucose uptake with similar potency and efficacy to isoprenaline, but the partial agonist has much weaker effects on cAMP accumulation and does not cause GRK phosphorylation, interactions with β-arrestin, receptor internalization, or desensitization. However, GRKs are important for glucose uptake as siRNA knock-down of GRK2 or GRK inhibitors decreased isoprenaline-stimulated glucose uptake. We conclude that the C-terminus of the β_2-AR is required

for interaction with GRK2 that is necessary for GLUT4 translocation and glucose uptake in response to agonist activation of the receptor. Weak partial agonists for cAMP accumulation such as BRL37344 have potential as therapeutic agents for the treatment of diabetes by increasing glucose uptake into skeletal muscle associated with minimal side effects and no propensity for desensitization.

AUTHOR CONTRIBUTIONS

SH, SM, MS, PK, and DSH performed the experiments. DSH, RJS, BAE, NDH, SF, and TB contributed to the interpretation of the results. DSH, RJS, and BAE designed the study. DSH, BAE, TB, and NDH supervised PhD student SM. SH, DSH, BAE, and RJS wrote the manuscript with input from TB and NDH.

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CONFLICT OF INTEREST STATEMENT

TB owns stocks in the following pharmaceutical companies: Sigrid Therapeutics AB, Atrogi AB, and Glucox Biotechnology AB. DSH, NDH, and RJS are consultants for Atrogi AB.

DATA AVAILABILITY STATEMENT

All data is available by request.

ETHICS STATEMENT

Approval from an institutional review board is not applicable.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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