RESEARCH ARTICLE | Obesity, Diabetes and Energy Homeostasis

BRL37344 stimulates GLUT4 translocation and glucose uptake in skeletal muscle via \( \beta_2 \)-adrenoceptors without causing classical receptor desensitization

Saori Mukaida,1,* Masaaki Sato,1,* Anette I. Öberg,2 Nodi Dehvari,2 Jessica M. Olsen,2 Martina Kocan,1 Michelle Louise Halls,1 Jon Merlin,1 Anna L. Sandström,2 Robert I. Csikasz,2 Bronwyn Anne Evans,1 Roger James Summers,1,3 @ Dana Sabine Hutchinson,1,2 and Tore Bengtsson2

1Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria, Australia; 2Department of Molecular Biosciences, The Wenner-Gren Institute, The Arrenius Laboratories F3, Stockholm University, Stockholm, Sweden; and 3Department of Pharmacology, Monash University, Clayton, Victoria, Australia

Submitted 13 September 2018; accepted in final form 8 March 2019

Mukaida S, Sato M, Öberg AI, Dehvari N, Olsen JM, Kocan M, Halls ML, Merlin J, Sandström AL, Csikasz RI, Evans BA, Summers RJ, Hutchinson DS, Bengtsson T. BRL37344 stimulates GLUT4 translocation and glucose uptake in skeletal muscle via \( \beta_2 \)-adrenoceptors without causing classical receptor desensitization. Am J Physiol Regul Integr Comp Physiol 316: R666–R677, 2019. First published March 20, 2019; doi:10.1152/ajpregu.00285.2018.—The type 2 diabetes epidemic makes it important to find insulin-independent ways to improve glucose homeostasis. This study examines the mechanisms activated by a dual \( \beta_2 \)/\( \beta_3 \)-adrenoceptor agonist, BRL37344, to increase glucose uptake in skeletal muscle and its effects on glucose homeostasis in vivo. We measured the effect of BRL37344 on glucose uptake, glucose transporter 4 (GLUT4) translocation, cAMP levels, \( \beta_2 \)-adrenoceptor desensitization, \( \beta \)-arrestin recruitment, Akt, AMPK, and mammalian target of rapamycin (mTOR) phosphorylation using L6 skeletal muscle cells as a model. We further tested the ability of BRL37344 to modulate skeletal muscle glucose metabolism in animal models (glucose tolerance tests and in vivo and ex vivo skeletal muscle glucose uptake). In L6 cells, BRL37344 increased GLUT4 translocation and glucose uptake only by activation of \( \beta_2 \)-adrenoceptors, with a similar potency and efficacy to that of the nonselective \( \beta \)-adrenoceptor agonist isoprenaline, despite being a partial agonist with respect to cAMP generation. GLUT4 translocation occurred independently of Akt and AMPK phosphorylation but was dependent on mTORC2. Furthermore, in contrast to isoprenaline, BRL37344 did not promote agonist-mediated desensitization and failed to recruit \( \beta \)-arrestin1/2 to the \( \beta_2 \)-adrenoceptor. In conclusion, BRL37344 improved glucose tolerance and increased glucose uptake into skeletal muscle in vivo and ex vivo through a \( \beta_2 \)-adrenoceptor-mediated mechanism independently of Akt. BRL37344 was a partial agonist with respect to cAMP, but a full agonist for glucose uptake, and importantly did not cause classical receptor desensitization or internalization of the receptor.

\( \beta_2 \)-adrenoceptor; \( \beta \)-arrestin; BRL37344; glucose uptake; GLUT4; isoprenaline; receptor desensitization; skeletal muscle

INTRODUCTION

Worldwide there are currently 425 million adults living with type II diabetes (T2D), with the incidence to increase by 48% to 629 million by 2045 (https://diabetesatlas.org/). The primary defects involved in the development and progression of T2D are decreased peripheral glucose utilization due to loss of insulin sensitivity, impaired pancreatic insulin secretion, and increased hepatic glucose production (3). Skeletal muscle is the primary site of insulin-mediated glucose uptake in the body, accounting for ~80% of glucose clearance (16). While current medications such as metformin increase peripheral insulin sensitivity, they are not without side effects, including lactic acidosis, and this approach has not been recommended for patients with impaired liver and kidney function, although recently these restrictions have been reduced (10). Since the insulin signaling pathway is impaired in T2D (18), the identification of new therapeutic avenues that target peripheral glucose utilization independently of insulin would have considerable merit.

Over 30 G protein-coupled receptors (GPCRs) have been implicated in the development, progression, and treatment of insulin resistance, obesity, and T2D (38), including the \( \beta_2 \)-adrenoceptor (\( \beta_2 \)-AR) in skeletal muscle. Activation of the \( \beta_2 \)-AR either by the endogenous ligands epinephrine and norepinephrine, or by selective \( \beta_2 \)-AR agonists, causes an increase in glucose uptake (1, 28, 32, 33, 41). One drawback of this approach is that \( \beta_2 \)-ARs are also expressed in many other tissues including lung, heart, blood vessels, kidney, brain, lymphocytes, and liver, potentially giving rise to a multitude of side effects. Additionally, glucose homeostasis may also be affected since \( \beta_2 \)-AR activation is known to increase the secretion of glucagon and insulin (2, 24) and to stimulate hepatic gluconeogenesis and glycolysis (15, 45). Persistent exposure of \( \beta_2 \)-ARs to agonists can also result in desensitization, suggesting that any approach attempting to target \( \beta_2 \)-ARs therapeutically to promote glucose uptake must minimize effects on tissues other than skeletal muscle and the loss of efficacy through receptor desensitization.

In skeletal muscle, almost all \( \beta_2 \)-AR-mediated effects are thought to be attributable to cAMP, including inhibition of proteolysis (31), increased ion transport (17), increased force of contraction (7), and changes in gene expression (30). However, the signaling pathways contributing to \( \beta_2 \)-AR-mediated glucose uptake have until recently been unclear. In contrast, insulin increases glucose uptake utilizing a well-characterized pathway. Activation of the insulin receptor and insulin receptor
substrate (IRS) proteins causes increased phosphoinositide 3-kinase (PI3K) activity and promotes phosphatidylinositol (3–5)-trisphosphate (PIP3) formation at the inner plasma membrane, which in turn recruits inactive Akt and 3-phosphoinositide-dependent protein kinase-1 (PKD1) to the cell surface. This facilitates PKD1-mediated phosphorylation of Akt at Thr308, causing a conformational change in Akt required for subsequent phosphorylation at Ser473 and full activation, leading to phosphorylation of AS160 and translocation of glucose transporter 4 (GLUT4) to the plasma membrane. Initial studies suggesting that PI3K, a key mediator of insulin-stimulated glucose uptake, was involved in β2-AR-mediated glucose uptake were based on the use of pharmacological inhibitors such as wortmannin and LY294002 (33, 41). However, these PI3K inhibitors were likely mediating their effects by inhibition of related kinases, since a downstream target of PI3K, Akt, was not stimulated by activation of the β2-AR (33, 41). Our recent work has revealed that β2-AR-stimulated glucose uptake in skeletal muscle involves mammalian target of rapamycin (mTOR) and subsequent translocation of GLUT4 from intracellular vesicles to the plasma membrane (41). While cAMP analogs mimic the effects of β2-AR agonists and also increase mTOR phosphorylation, GLUT4 translocation, and glucose uptake, inhibition of the Gαs-cAMP pathway only partially inhibits β2-AR-mediated glucose uptake (33, 41). As such, the underlying mechanism whereby β2-AR activation increases mTOR activity and GLUT4 translocation is still unclear.

While β2-ARs classically couple to Gαs, leading to increases in intracellular cAMP, it is now clear that β2-AR signaling is pleiotropic, with downstream effects also mediated through Gαs/o proteins, MAP kinases, GPCR kinases, and β-arrestins (14). There is growing evidence to suggest that receptors, such as the β2-AR, that activate pleiotropic signaling may respond to different ligands with preferential activation of pathways giving rise to different signaling profiles, termed biased signaling (14). This is important, since β2-AR agonists such as clenbuterol increase skeletal muscle glucose uptake in vitro and in vivo and improve glucose tolerance in vivo (34, 35, 41), yet its use in humans is problematic due to side effects including skeletal muscle tremor and cardiovascular complications. Therefore, we aimed to determine if there are ligands that activate the β2-AR to increase glucose uptake but cause minimal cAMP signaling, to minimize off-target side effects in muscle, heart, and liver. Here, we suggest that another ligand, the dual β2/β3-AR agonist BRL37344, may have more potential. BRL37344 was originally developed in rodent models as a β3-AR agonist (4) for the treatment of obesity but failed to show efficacy in clinical trials. However, studies in humans showed that it did improve glucose tolerance and insulin sensitivity in obese and diabetic patients (8). It was subsequently shown in some tissues to be a full β2-AR agonist, increasing glucose uptake in skeletal muscle solely through actions at β2- and not β3-ARs (27, 32, 34, 35). Here we find that in L6 skeletal muscle cells, BRL37344 has little effect on cAMP production but is as effective as isoprenaline and insulin in promoting GLUT4 translocation and glucose uptake, suggesting that BRL37344 may have beneficial signaling properties at the β2-AR compared with classical β2-AR agonists such as isoprenaline. In addition, BRL37344, unlike classical high efficacy β2-AR agonists, increased glucose uptake without causing β2-AR desensitization. Finally, activation of β2-ARs by BRL37344 had beneficial effects in vivo in glucose intolerant mice.

**MATERIALS AND METHODS**

**Cell culture.** L6 cells from American Type Culture Collection (Manassas, VA) or L6 cells overexpressing GLUT4 with a myc-epitope (L6GLUT4myc; kindly provided by Amira Klip, Hospital for Sick Children, Toronto, Canada) were grown in DMEM supplemented with 4 mM l-glutamine, 10% (vol/vol) FBS, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES, in a 37°C incubator with 5% CO2. Cells were grown as myoblasts by ensuring cells were kept at <70% confluency. Upon confluency (90%), differentiation was induced by lowering the FBS concentration to 2% (vol/vol) for 7 days, with media changes every 2 days. Before the experiment, L6 cells were serum starved overnight. Human primary skeletal muscle cells (SKMCs) were purchased from KaroCell (Stockholm, Sweden), Lonza (Basel, Switzerland), and Promocell (Heidelberg, Germany) and grown in Ham's F-10 media containing 20% (vol/vol) heat-inactivated FBS, 2 mM l-glutamine, 50 μg/ml penicillin, and 50 μg/ml streptomycin. While SKMCs were obtained from different companies, there was no difference between responses from the differently sourced human cells (data not shown). Differentiation was initiated by reducing FBS levels to 4% (vol/vol) for 3 days followed by 2% (vol/vol) for 4 days. Before the experiment, SKMCs were serum starved overnight. CHO-K1 cells stably transfected with the human GLUT4 with a myc-epitope (CHOGLUT4myc cells; Ref. 22) were grown in 50:50 DMEM/Ham’s F12 medium supplemented with 5% (vol/vol) FBS at 37°C with 5% CO2.

**cAMP assays.** Agonist-stimulated cAMP accumulation in L6 cells was measured using the LANCE cAMP detection kit (PerkinElmer) with 384-well white Optiplates (PerkinElmer) and a 2103 EnVision plate reader (PerkinElmer). L6 cells were plated at 10,000 cells/well in 96-well plates and differentiated as described above. Cells were stimulated with BRL37344 or isoprenaline in stimulation buffer [HBSS containing 0.1% (wt/vol) bovine serum albumin (BSA), 5 mM HEPES, 1.3 mM CaCl2, and 5.6 mM glucose, pH 7.4] for indicated times at 37°C. The assay was terminated by addition of 50 μl/well of 99.8% ethanol, and samples were evaporated overnight. Lysis buffer (90.1% [wt/vol] BSA, 0.3% [vol/vol] Tween-20, and 5 mM HEPES, pH 7.4; 100 μl/well) was added to each well. cAMP levels were quantified by mixing 10 μl of cell lysate with 5 μl cAMP antibodies labeled with Alexa Fluor 647 (ThermoFisher Scientific) and incubating for 30 min at room temperature. Ten microliters of detection solution containing biotin-cAMP and europium-labeled streptavidin were added and incubated overnight. Time-resolved Förster resonance energy transfer (FRET) signals were acquired in a 2103 EnVision plate reader (PerkinElmer) using excitation at 340 nm, with emission measured at 615- and 665-nm wavelengths.

**FRET assays.** To examine whether BRL37344 and isoprenaline caused localized production of cAMP in different regions of the cell, L6 myoblasts were transfected with either the cAMP biosensor pmEpac2 that detects cAMP at the cell membrane or with cytoEpac2 to detect cAMP in the cytoplasm (19). L6 myoblasts were plated at a density of 10,000 cells/well in black optically clear 96-well microplates (PerkinElmer) as described above. Cells were transfected with 40 ng of the FRET biosensors pmEpac2 or cytoEpac2 (36, 46) using 1 mg/ml polyethylenimine. Twenty-four hours after transfection, cells were changed to DMEM containing 0.5% FBS (vol/vol) overnight.

Single live cell FRET experiments were performed using a high-content GE Healthcare Incell 2000 Analyzer as described previously (19). Briefly, fluorescence imaging was performed with a Nikon Plan Fluor ELWD ×40 (numerical aperture, 0.6) objective and FRET module. Cells were sequentially excited with a CFP filter (430/24) with emission measured with YFP (535/30) and CFP (470/24) filters.
and a polychroic mirror optimized for the CFP/YFP filter pair (Quad3). L6 cells in HBSS at 37°C were measured every 1 min for 4 min to generate baseline emission ratio values. Cells were stimulated with vehicle or ligand, and images were captured every 1 min for 20 min. At the end of each experiment, the same cells were stimulated for 10 min with the positive control (10 μM forskolin and 100 μM 3-isobutyl-1-methylxanthine) to generate a maximal FRET change, and the positive emission ratio images were captured for 4 min. Data were analyzed with the FIJI distribution of ImageJ (42). The three emission ratio image stacks (baseline, stimulated, and positive) were collated and aligned with the StackCreator script (19). Cells were selected with the CellMarkup script (19), and fluorescence intensity was measured over the combined stack. Background intensity was subtracted, and then, the FRET data were plotted as the change in FRET emission ratio relative to the maximal response for each cell.

Real-time kinetic bioluminescence resonance energy transfer assays. CHO/CLTr-f4-myc cells seeded in six-well plates at a density of 600,000 cells/well were cotransfected with β3AR-Rluc8 (100 ng/6-well) and ß-arrestin-1-Venus (300 ng/6-well), ß-arrestin-2-Venus (300 ng/6-well) or Kras-Venus (300 ng/6-well) using Lipofectamine 2000 (Invitrogen). The next day, cells were replated in white tissue culture-treated 96-well microplates and grown overnight in phenol red-free medium (supplemented with 4 mML-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES). Cells were treated with BRL37344 (10 μM), isoprenaline (1 μM), or insulin (1 μM) for 2 h. Media were discarded, and cells fixed with 2% (wt/vol) formaldehyde in PBS for 15 min and washed with PBS three times. Then, cells were quenched with 150 mM Tris (pH 8.0) for 10 min, and washed with PBS three times. Cells were then blocked with 5% (wt/vol) BSA in PBS for 1 h, washed with PBS three times, and incubated with Myc-tag primary antibody (cat. no. 2272; Cell Signaling Technology) solution (1:50 dilution in 1.5% (wt/vol) BSA in PBS) overnight at 4°C. Cells were then washed with PBS and incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (cat. no. A-11008; ThermoFisher Scientific, Scoresby, VIC, Australia) (1:1,000 dilution and 1.5% BSA in PBS) for 1 h. Nuclei were stained by DAPI (5 μg/ml) for 3 min. Images were observed in a Leica DMLB epifluorescence microscope. Images were acquired using a DC350F camera with IM500 software (Leica Microsystems).

Glucose uptake in L6 and SKMCs. Glucose uptake was measured as previously described (32) with changes in response to drug addition measured as percent control. Briefly, cells were plated at 100,000 cells/well in 12-well plates and differentiated as described above. Cell stimulation was performed with antagonists (1 μM SR59230A; 1 μM ICI118551) or inhibitors (1 μM KU0063794; 1 μM Compound C) added 30 min ahead of agonists that were then incubated for 2 h at 37°C. Medium was then exchanged to glucose-free medium, drugs were readded, and plates were incubated for a further 15 min before 2-deoxy-d-[3H]glucose (50 nM) was added and the plates incubated for a further 15 min. Reactions were terminated by being washed twice in ice-cold phosphate-buffered saline (PBS). Cells were digested (0.2 M NaOH, 1 h, 60°C), and samples were transferred to vials with scintillation liquid and radioactivity measured by liquid scintillation counting.

Western blotting. Differentiated myotubes in 12-well plates were serum starved overnight before the experiment and were exposed to drugs for times and concentrations indicated with the data. Cells were lysed directly by the addition of 65°C lysis buffer (62.5 mM Tris pH 6.8, 2% wt/vol sodium dodecyl sulfate, 10% vol/vol glycerol, 50 mM dithiothreitol, and 1% wt/vol bromphenol blue). The lysate was sonicated briefly followed by boiling for 3 min. Aliquots of samples (of same protein amount) were separated on 8% or 12% polyacrylamide gels and electrotransferred to Hybond-P polyvinylidene fluoride membranes (pore size: 0.45 μm; GE Healthcare). Primary antibodies (total AKT; cat. no. 9272), phospho-CRE binding protein (CREB) Ser 133 (cat. no. 9198), phospho-AMP-activated protein kinase (AMPK) Thr 172 (cat. no. 2535), total AMPK (cat. no. 5831), and mTOR Ser2481 (cat. no. 2974) diluted 1:1,000 were all from Cell Signaling and were detected using a secondary antibody (horseradish peroxidase-linked anti-rabbit IgG; cat. no. 7074; Cell Signaling, Danvers, MA) diluted 1:2,000 and ECL (GE Healthcare). The blots were exposed to film (GE Healthcare) and quantified on a Molecular Dynamics densitometer using ImageQuant NT software. Antibodies for immunoblotting were validated by the appearance of a single band at the known/predicted molecular weight for the protein of interest and, in the case of phospho-proteins, an increase in their levels by a known positive control (data not shown).

αScreen assays for protein phosphorylation. Phosphorylation of Akt1,2,3 (Thr308/Ser473) and S6RP (Ser235/236) was measured using αScreen SureFire kits (PerkinElmer). L6 cells were seeded in 96-well plates at a density of 10,000 cells per well, differentiated as described above, serum starved overnight, and then stimulated with BRL37344, isoprenaline, and insulin for the indicated time periods. Protein phosphorylation was measured and detected according to the manufacturer’s instructions.

Immunocytochemistry and immunofluorescence. L6GLUT4-myc cells were plated at 10,000 cells/well in eight-well culture chamber slides (BD Biosciences, Franklin Lakes, NJ) and differentiated as described above. After being serum starved overnight in DMEM (supplemented with 4 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES), cells were treated with BRL37344 (10 μM), isoprenaline (1 μM), or insulin (1 μM) for 2 h. Media were discarded, and cells fixed with 2% (wt/vol) formaldehyde in PBS for 15 min and washed with PBS three times. Then, cells were quenched with 150 mM Tris (pH 8.0) for 10 min, and washed with PBS three times. Cells were then blocked with 5% (wt/vol) BSA in PBS for 1 h, washed with PBS three times, and incubated with Myc-tag primary antibody (cat. no. 2272; Cell Signaling Technology) solution (1:500 dilution in 1.5% (wt/vol) BSA in PBS) overnight at 4°C. Cells were then washed with PBS and incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (cat. no. A-11008; ThermoFisher Scientific, Scoresby, VIC, Australia) (1:1,000 dilution and 1.5% BSA in PBS) for 1 h. Nuclei were stained by DAPI (5 μg/ml) for 5 min. Images were observed in a Leica DMLB epifluorescence microscope. Images were acquired using a DC350F camera with IM500 software (Leica Microsystems).

Animals. All studies were approved by the North Stockholm Animal Ethics Committee. β3-Adrenergceptor knockout (β3-KO) mice on FVB/N background (43) were bred at Stockholm University (Stockholm, Sweden). A total number of 28 mice were used in this study. For in vivo and ex vivo glucose uptake experiments, 12- to 16-wk-old female β3-AR KO mice fed a chow diet were used. For the in vivo glucose tolerance tests, female β3-AR KO mice were fed a high-fat diet at 12 wk of age (45% kcal fat, 35% kcal carbohydrates, 20% kcal proteins; 4.7 kcal/g; cat. no. D12451; Research Diets) for 14 wk. Mice were intraperitoneally injected with 1 g/kg of D-Glucose (Sigma-Aldrich) after an overnight fast. After 30 min, glucose levels were measured in tail blood samples.

Animal Ethics Committee.

In vivo glucose uptake. In vivo glucose uptake was measured using the 2-deoxy-d-[3H]glucose method (28) with some modifications. Mice were fasted for 5 h and anesthetized with 67 mg/kg pentobarbital ip and once anesthetized were injected with BRL37344 (1 mg/kg ip) or saline. In total, 12 mice were used (6 treated with saline and 6 treated with BRL37344). After 20 min, 2-deoxy-d-[3H]glucose (130 μCi/kg; Perkin Elmer, Waltham, MA; 8 Ci/mmole) was injected intraperitoneally, and mice were killed by CO2 1 h later. Skeletal muscle was dissected and lysed in 0.5 M NaOH for 1 h, and radioactivity was measured by liquid scintillation counting.

Ex vivo glucose uptake in gastrocnemius and soleus muscle. Gastrocnemius or soleus muscles were dissected from β3-KO mice and suspended in Krebs-Henseleit bicarbonate (KHB) buffer (118.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 25 mM NaHCO3, 2.5 mM
CaCl2, 1.2 mM MgSO4, and 5 mM HEPES) in organ baths containing 30 ml KHB buffer with 5 mM glucose and 15 mM mannitol, bubbled with 95% O2-5% CO2 (pH 7.4) and maintained at 37°C. Muscle was incubated with BRL37344 for 1 h, before being rinsed with KHB buffer (containing 20 mM mannitol) for 10 min, followed by incubation in KHB buffer [containing 8 mM 3-O-methylglucose, 12 mM mannitol, 438 µCi/mmol 3-O-methyl[14C]glucose (80.2 Ci/mmol; PerkinElmer), and 42 µCi/mmol [13C]mannitol (58.8 mCi/mmol; PerkinElmer)] for 12 min. Muscles were rinsed with PBS and frozen in liquid nitrogen before being weighed and digested in 1 ml of 0.5 M NaOH at 60°C. 1H and 14C were measured by liquid scintillation counting. Total muscle 3-O-methylglucose and extracellular space were measured as described previously (48). Intracellular 3-O-methylglucose accumulation was calculated by the following: total muscle 3-O-methylglucose - extracellular 3-O-methylglucose = intracellular 3-O-methylglucose. This is then expressed as a rate of 3-O-methylglucose transport per milliliter of intracellular water per hour. In total, six mice were used, with soleus and gastrocnemius muscle taken from the same animal, and muscles from both hindlimbs were used.

In vivo glucose tolerance test. Mice were treated with 1 mg/kg BRL37344 ip twice a day for 4 days before in vivo glucose tolerance tests were performed. In total, 10 mice were used (5 treated with saline and 5 treated with BRL37344). On the fifth day, mice were fasted for 6 h before glucose (2 g/kg ip) administration. Blood glucose was measured from a small cut in the tail vein using a glucometer (Accu-Check Aviva) before glucose injection and following 15, 30, 60, and 90 min after glucose addition.

Data analysis. All measurements were taken in duplicate-quadruplicate with n referring to the number of independent experiments performed, and the results are expressed as the means ± SE. The statistical significance of differences between groups was analyzed by either Student’s t-test or one- or two-way ANOVA, followed by post hoc Dunnett or Tukey multiple comparison tests when appropriate. Statistical significance was set at P < 0.05.

RESULTS

Glucose uptake in skeletal muscle in response to isoprenaline and BRL37344 is mediated by β2-adrenoceptors. In L6 myotubes, the prototypical β-AR agonist isoprenaline (pEC50 7.3 ± 0.2; maximal response 175 ± 3.5%), the dual β2/β3-AR agonist BRL37344 (pEC50 6.9 ± 0.1; maximal response 162 ± 3.2%), and insulin (pEC50 7.3 ± 0.2; maximal response 170 ± 3.7%), all increased glucose uptake in a concentration-dependent manner (Fig. 1A). These responses were associated with significant increases of GLUT4 localization at the cell surface (Fig. 1, B and C), measured in L6 cells stably expressing GLUT4myc. BRL37344, isoprenaline, and insulin also significantly increased glucose uptake in human SKMC myotubes (Fig. 1D). In L6 cells, the selective β3-AR agonist CL316243 (0.1–10 µM) did not affect glucose uptake (Fig. 1E), and the β3-AR antagonist SR59230A had no significant effect on glucose uptake stimulated by BRL37344, isoprenaline, or insulin (Fig. 1F). This suggests that stimulation of glucose uptake by BRL37344 in L6 cells did not involve β3-ARs, that in addition are not expressed in L6 cells (32). In contrast, responses to BRL37344 and isoprenaline (but not insulin) in L6 cells were blocked by the β3-AR selective antagonist ICI118551 (Fig. 1G), indicating that glucose uptake requires the β2-AR.

Effect of BRL37344 on glucose uptake in skeletal muscle ex vivo and in vivo and on glucose tolerance in vivo. The effect of BRL37344 on glucose uptake in skeletal muscle ex vivo and in vivo was assessed in β3-AR KO mice to remove any possible contribution of β3-ARs to the BRL37344 response. In gastrocnemius and soleus muscle isolated from β3-AR KO mice, BRL37344 significantly increased glucose uptake (Fig. 2A). Acute administration of BRL37344 in vivo also significantly increased glucose uptake into gastrocnemius and soleus muscle in β3-AR KO mice (Fig. 2B). To assess whether this effect of BRL37344 was retained in a diabetic model, we used β3-AR KO mice fed a high-fat diet at thermoneutrality (30°C) for 14 wk and then treated twice a day for 4 days with either saline or BRL37344 (1 mg/kg ip). Significant improvements in glucose tolerance were observed in mice treated with BRL37344 (Fig. 2, C and D).

Isoprenaline, but not BRL37344, produces robust increases in cAMP levels in rat skeletal muscle L6 cells. cAMP accumulation was measured following stimulation of L6 cells with isoprenaline or BRL37344 in the absence of phosphodiesterase inhibitors. Examination of the time course of cAMP accumulation showed a peak cAMP response after 5 min of stimulation with isoprenaline (1 µM) and BRL37344 (10 µM); however, the maximal response to isoprenaline was ~3.3-fold higher (P < 0.001) compared with that of BRL37344 (Fig. 3A). BRL37344 was also significantly less potent than isoprenaline (pEC50 6.7 ± 0.1 and 8.4 ± 0.2 respectively; P < 0.001) (Fig. 3B). A downstream target of cAMP is phosphorylation of CREB. Whereas isoprenaline (1 µM) administration increased CREB phosphorylation levels by approximately threefold after 30 min of stimulation (Fig. 3C), BRL37344 (10 µM) failed to produce a significant increase in CREB phosphorylation at any time point (Fig. 3C). Both isoprenaline (1 µM) and BRL37344 (10 µM) increased cAMP levels at the plasma membrane and in the cytoplasm (using the cAMP biosensor pmEpac2 or cytoEpac2, respectively), with the response to isoprenaline significantly higher (P < 0.001) than that to BRL37344 (Fig. 3, D and E).

Increased glucose uptake in rat skeletal muscle L6 cells in response to isoprenaline and BRL37344 involves mTORC2 but not activation of Akt or AMPK. Insulin (1 µM) caused phosphorylation of Akt at Thr308 and Ser473, in contrast to isoprenaline (1 µM) and BRL37344 (10 µM), which produced no change (Fig. 4, A and B). Likewise, 5-aminimidazole-4-carboxamide ribonucleotide (AICAR, 2 mM) caused phosphorylation of AMPK at Thr172, whereas isoprenaline (1 µM) and BRL37344 (10 µM) produced no change (Fig. 4C). In addition, glucose uptake in response to BRL37344, isoprenaline, or insulin was not affected by the AMPK inhibitor compound C (1 µM) (Fig. 4D). However, glucose uptake in response to BRL37344, isoprenaline, and insulin was inhibited by the selective mTOR inhibitor KU0063794 (1 µM) suggesting that glucose uptake involved mTOR (Fig. 5A). Since BRL37344 and isoprenaline both caused phosphorylation of mTOR at Ser2481, it is likely that mTORC2 rather than mTORC1 is involved (Fig. 5B). BRL37344 did not phosphorylate the mTORC1 downstream target S6RP, whereas isoprenaline caused a small but significant increase in S6RP phosphorylation at 60 and 120 min (Fig. 5C).

Isoprenaline, but not BRL37344, recruits β-arrestin to the receptor, resulting in receptor desensitization. To determine whether isoprenaline or BRL37344 causes desensitization of β2-AR-mediated responses, we measured cAMP accumulation following preexposure of L6 cells to either BRL37344 (10 µM) or isoprenaline (1 µM). Preexposure to BRL37344 failed
to desensitize subsequent cAMP responses to either BRL37344 or isoprenaline, whereas preexposure to isoprenaline desensitized subsequent responses to either agonist (Fig. 6, A–D). In addition, L6 cells exposed to either isoprenaline or BRL37344 for periods of up to 20 hrs were then tested to determine glucose uptake after a 15 min incubation with 2-deoxy-D-[3H]glucose. Glucose uptake was slightly inhibited after prolonged exposure to isoprenaline but not BRL37344 (Fig. 6, E).

We next examined the effect of β2-AR activation by isoprenaline and BRL37344 on BRET between β2-AR Rluc-8 and both β-AR antagonist ICI118551 (1 μM) significantly inhibited glucose uptake in response to BRL37344 (10 μM) and isoprenaline (1 μM) but not that to insulin (1 μM), in L6 myotubes (n = 6–10). *P < 0.05, **P < 0.01, ***P < 0.001, statistical difference as analyzed by one-way ANOVA between control and treated samples.

DISCUSSION

BRL37344, and BRL35135 (which is deesterified in vivo to BRL37344) were originally developed as antiobesity drugs targeting the β3-AR (4), with studies showing that BRL35135 caused a decrease in body weight and whole body fat composition in female ob/ob mice (4, 8) and decreased weight gain in genetically obese male Zucker rats (39), independently of
effects on food intake (4, 8, 39). These antiobesity effects did not translate into humans, with BRL35135 having no effect on body weight when administered to obese subjects for 10 days (8, 29). However, BRL37344 and BRL35135 did have favorable effects on glucose homeostasis both in humans and rodents. In humans, BRL35135 improved glucose tolerance and increased insulin-mediated glucose disposal, with no effects on fasting blood glucose or plasma insulin levels (8, 29). In obese rats, chronic BRL35135 administration improved glucose tolerance and lowered plasma insulin levels (8, 39) and increased skeletal muscle glucose uptake in lean rats (28). Acute BRL35135 administration also increased glucose uptake into skeletal muscle (1, 28). This collective data clearly show that BRL37344 and BRL35135 improve glucose homeostasis in both rodents and humans.

Responses to BRL37344 in skeletal muscle are mediated through β2-ARs, based on studies in L6 skeletal muscle cells that show that β2- but not β3-ARs are expressed, on pharmacological studies using receptor-specific antagonists (32), and on glucose uptake responses in vivo in β3-AR KO mice (28). Our study here utilized L6 cells and also β3-AR KO mice to exclude any actions BRL37344 may have on β3-ARs expressed in other metabolically active tissues such as adipose tissue. In L6 cells, isoprenaline, BRL37344, and insulin had similar efficacy in promoting glucose uptake and in promoting GLUT4 translocation to the cell surface. All three drugs also promoted glucose uptake in human SKMCs. We confirmed that glucose uptake in L6 cells was due to actions on β2-AR since the effects of isoprenaline and BRL37344 were blocked by the β2-AR-selective antagonist ICI118551 but not the β3-AR selective antagonist SR59230A. BRL37344 also increased glucose uptake both ex vivo and in vivo in gastrocnemius and soleus muscles from β3-AR KO mice. In addition, chronic treatment with BRL37344 improved glucose tolerance in β3-AR KO mice placed on a high-fat diet for 14 wk. Theoretically on-target actions of BRL37344 at the β3-AR would not be detrimental to its use in vivo since β3-ARs are expressed in human adipose tissue (in particular brown adipose tissue (13) and the gastrointestinal tract/urogenital system, with similar efficacy and potency to mirabegron (44). With respect to actions in adipose tissue, in healthy adult humans, mirabegron increases brown adipose tissue activity (11), and brown adipose tissue plays a major role in cold-induced glucose clearance in mice (6). Hence, any actions that BRL37344 may have at β3-ARs in human tissues would be beneficial, especially in the context of individuals with T2D. However, BRL37344 has been shown to be ineffective in human white adipocytes (25), and clinical trials using the pro-drug BRL35135 showed no effects on body weight or thermogenesis (8, 29). There are no studies examining the effects of BRL37344/35135 in human brown adipose tissue or brown adipocytes, making it unclear if it has any potential effect on brown fat function, although in obese humans BRL35135...
Acutely increases energy expenditure (8). The mechanism whereby this occurs is unknown. It is most likely that beneficial effects of BRL37344 on glucose homeostasis in humans occurs through interactions at β/2-ARs.

A second question is whether BRL37344 activation of β/2-ARs has adverse effects in cell types other than skeletal myocytes, especially cardiomyocytes. We have shown here, using a variety of techniques, that while BRL37344 is a full agonist for glucose uptake, it is a partial agonist for cAMP accumulation. Direct measurement of cAMP, pCREB activation, and the use of cell membrane and cytoplasmic cAMP biosensors all showed weaker responses to BRL37344 than isoprenaline. Despite this, cardiac responses to BRL37344/BRL35135 have been observed. In rodents, BRL37344 was a partial agonist (~80% of the response to isoprenaline) for chronotropic effects due to actions at β/1/β/2-ARs (4, 47). These effects occur at higher concentrations than those required for lipolysis in white and brown adipocytes (BRL35135 is 33-fold and BRL37344 more than 400-fold selective for stimulating lipolysis vs. atria rate; Refs. 4, 47). In human atria, BRL37344 exhibited partial agonist effects (~40% of the maximal response to isoprenaline) for inotropic responses that were an-

Fig. 3. A: time course of cAMP accumulation in response to isoprenaline (1 μM) and BRL37344 (10 μM) in rat L6 myotubes (n = 6–8) (Basal = 0.47 pmol cAMP/well). Experiments were performed in the absence of phosphodiesterase inhibition. B: isoprenaline and BRL37344 increased intracellular cAMP levels in a concentration-dependent manner in rat L6 myotubes (n = 7) after 30 min of stimulation with either isoprenaline or BRL37344. C: effect of isoprenaline (1 μM) or BRL37344 (10 μM) on CRE binding protein (CREB) phosphorylation levels in rat L6 myotubes. Blot is a representative image from 3 experiments. D and E: effect of isoprenaline (1 μM) or BRL37344 (1 μM) on cAMP levels over time in L6 myoblasts transfected with the cAMP biosensor pmEpac2 to detect cAMP at the cell membrane (D) or cytoEpac2 to detect cAMP in the cytoplasm (n = 5) (E). **P < 0.01, ***P < 0.001, statistical difference as analyzed by two-way ANOVA (C) or two-way repeated measure ANOVA (D and E) between control and treated samples.
agonized by propranolol (37). These responses to BRL37344 were observed only at concentrations \( \leq 1 \text{ M} \) (37). When BRL35135 was administered to healthy male subjects, an increase in heart rate (\( \sim 9 \) beats/min) was observed (8). However, it should be noted that the individuals who displayed the greatest improvements in glucose tolerance and decreases in hyperinsulinaemia were those that were the most glucose intolerant/diabetic and that no arrhythmias, tachycardia, or changes in blood pressure were noted. The only visible side effect was a mild tremor in the fingers/hands that was correlated with plasma BRL35135 levels (8). Thus, while retaining activity for glucose uptake in skeletal muscle, BRL37344 appears to exhibit relatively minor side effects in tissues where the response is mediated primarily by cAMP.

In skeletal muscle, \( \beta_2 \)-AR agonists (including BRL37344) increase glucose uptake by a pathway that is independent of the insulin signaling pathway (41). We confirmed that the response to \( \beta_2 \)-AR involves activation of mTORC2 (41) and showed that both \( \beta \)-AR agonists cause phosphorylation of mTOR at Ser2481 but also demonstrated that, in contrast to insulin, neither isoprenaline nor BRL37344 caused phosphorylation of Akt at Thr308 and Ser473 at Ser235/Ser236, known to be downstream of mTORC1, and BRL37344 had no effect on S6RP phosphorylation, suggesting together with phosphorylation of mTORC2 at Ser2481 that the dominant effect is on mTORC2. This is important since in T2D the insulin signaling pathway is downregulated mainly at the level of IRS1/PI3K/Akt activation (18), suggesting that increasing glucose uptake by a mechanism not involving this pathway could be attractive therapeutically. While the mechanistic details leading to \( \beta_2 \)-AR activation of mTORC2 are not clear, our previous studies indicate a partial role for cAMP (41), since isoprenaline-mediated mTORC2 phosphorylation was partially inhibited by a protein kinase A inhibitor (41), which suggests that other signaling mediators/effectors are involved. Whether

Fig. 4. A and B: insulin (1 \text{ M}), but not isoprenaline (1 \text{ M}) or BRL37344 (10 \text{ M}), increased phosphorylation of Akt at (A) Thr308 and (B) Ser473 in rat L6 myotubes \( (n = 4–6) \). C: 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR, 2 mM), but not isoprenaline (1 \text{ M}) or BRL37344 (10 \text{ M}), increased phosphorylation of AMPK at Thr172 in rat L6 myotubes \( (n = 4) \). D: glucose uptake in response to AICAR (2 mM) but not isoprenaline (1 \text{ M}), BRL37344 (10 \text{ M}), or insulin (1 \text{ M}), was significantly reduced by the AMPK inhibitor Compound C (1 \text{ M}) in rat L6 myotubes \( (n = 8) \). * \( P \leq 0.05 \) ** \( P \leq 0.01 \) *** \( P \leq 0.001 \), statistical difference as analyzed by one-way ANOVA (C) or two-way ANOVA (A and B) between control and treated samples.
BRL37344 requires cAMP or another effector to activate mTORC2 is not clear at this stage. Since both isoprenaline and BRL37344 are full agonists for glucose uptake but BRL37344 is only a partial agonist for cAMP, this may suggest that a small, localized increase in cAMP following acute BRL37344/isoprenaline treatment is sufficient to promote signaling pathways leading to GLUT4 translocation, as GLUT4myc abundance is rapidly altered. GLUT4 can then remain at the plasma membrane for a prolonged period (the protein half-life of GLUT4 is ~48 h; Ref. 40), with a single GLUT4 molecule being able to undergo multiple rounds of recycling before being targeted for degradation) to enable maximal glucose uptake. Thus there may be a difference in the rate of GLUT4 translocation, but at steady state, the rate of glucose uptake is similar in response to both isoprenaline and BRL37344. This concept is supported by our results showing marked desensitization of the cAMP response but only slight desensitization of glucose uptake after isoprenaline exposure, suggesting that a small amount of cAMP generated either by isoprenaline after desensitization or by BRL37344 is sufficient to promote GLUT4 translocation and glucose uptake. This concept requires further investigation.

Fig. 5. A: glucose uptake in response to isoprenaline (1 μM), BRL37344 (10 μM), and insulin (1 μM) was significantly reduced by the mammalian target of rapamycin (mTOR) inhibitor KU0063794 (1 μM) in rat L6 myotubes (n = 5–11). B: increased mTOR phosphorylation at Ser2481 after 2-h exposure to isoprenaline (1 μM) and BRL37344 (10 μM) in rat L6 myotubes (n = 5). C: insulin (1 μM) and isoprenaline (1 μM) but not BRL37344 (10 μM), significantly increased S6RP phosphorylation at Ser235/Ser236 compared with basal levels in rat L6 myotubes (n = 5–6). *P < 0.05, **P < 0.01, statistical difference as analyzed by one-way ANOVA between control and treated samples (A and B). *P < 0.05, **P < 0.01, ***P < 0.001, statistical difference as analyzed two-way ANOVA between control and treated samples (C).
Another important insulin-independent mechanism for increasing glucose uptake in skeletal muscle is activation of AMPK. Our previous results in L6 cells show that activation of $\beta_1$-ARs, and not $\beta_2$- or $\beta_3$-ARs or direct activation of adenylyl cyclase by forskolin, causes phosphorylation and activation of AMPK, which is required for $\beta_1$-AR-mediated glucose uptake (21). Since BRL37344 may increase glucose uptake through signaling pathways other than those utilized by isoprenaline, we examined whether BRL37344 caused phosphorylation of AMPK and whether BRL37344-mediated glucose uptake was inhibited by the AMPK inhibitor compound C. Our results clearly show that BRL37344 does not activate or require AMPK for increasing glucose uptake.

Receptor desensitization is a potential clinical problem associated with the long-term use of $\beta_2$-AR agonists. We therefore utilized several experimental paradigms to examine desensitization following isoprenaline or BRL37344 treatment. We showed that BRET interactions between the receptor and $\beta$-arrestin1 and 2 were increased by isoprenaline treatment and decreased between the receptor and the plasma membrane marker K-Ras. In contrast, BRL37344 treatment had no effect on any of the interactions. This is consistent with isoprenaline, but not BRL37344, causing receptor internalization, and isoprenaline, but not BRL37344 pretreatment, causing desensitization of $\beta_2$-AR signaling to subsequent $\beta_2$-AR agonism. This difference also translates to glucose uptake, which remained fully stimulated during continuous treatment (for 20 h) of L6 cells with BRL37344 but not to isoprenaline. The lack of desensitization to BRL37344 may be desirable clinically, as improvements in glucose homeostasis would require chronic administration.

Although BRL37344 is a weak partial agonist for cAMP production, and a full agonist for glucose uptake, we cannot at this stage conclude that BRL37344 is a biased agonist at the $\beta_2$-AR. Biased agonism has been demonstrated at the $\beta_2$-AR (14), with many studies focusing on the interaction of a GPCR with $\beta$-arrestins, leading to the concept of G$\beta$- or $\beta$-arrestin-biased compounds. Continuous activation of the $\beta_2$-AR by a full agonist leads to rapid receptor desensitization that occurs through a well-characterized pathway. Upon receptor activation, specific residues in the COOH-terminal tail are phosphorylated by GPCR kinases, leading to $\beta$-arrestin recruitment, receptor internalization, and a subsequent loss of $\beta_2$-AR responsiveness. In our study, BRL37344 stimulation of the $\beta_2$-AR failed to recruit $\beta$-arrestins, whereas isoprenaline promoted rapid recruitment. Thus BRL37344 is certainly not $\beta$-arrestin biased, and further studies will be needed to deter-
mine whether it promotes interaction of the β2-AR with other signaling proteins or complexes.

**Perspectives and Significance**

We have shown that BRL37344 increases glucose uptake into skeletal muscle by activation of β2-ARs in vitro, ex vivo, and in vivo. This involves increased translocation of GLUT4 to the cell surface by a mechanism involving activation of mTORC2 but utilizing a signaling pathway not involving Akt, clearly distinguishing the pathway from that promoted by insulin. Importantly, BRL37344 achieves this with minimal increases in cAMP and without recruiting β-arrestin to the receptor or receptor desensitization. The ability of BRL37344 to promote glucose uptake utilizing pathways other than those utilized by insulin that are desensitized in T2D may offer therapeutic opportunities. The results presented here suggest that targeting the β2-AR with agonists possessing these beneficial characteristics may provide a novel approach to the treatment of T2D.

**REFERENCES**

23. Liu YL, Cawthorne MA, Stock MJ. Bihaptic effects of the β-adrenoceptor agonist, BRL 37344, on glucose utilization in rat isolated skeletal
mechanisms of β3-adrenoceptor-mediated glucose uptake


