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Characterization of the β-adrenoceptor subtype involved in mediation of glucose transport in L6 cells

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1 The receptor that mediates the increase in glucose transport (GT) in response to β-adrenoceptor (β-AR) agonists was characterized in the rat skeletal muscle cell line L6, using the 2-deoxy-[^{3}H]-D-glucose assay.

2 The β_3-AR agonist BRL37344 ($pEC_{50} = 6.89 \pm 0.21$), the β-AR agonist isoprenaline ($pEC_{50} = 8.99 \pm 0.24$) and the β_2-AR agonist zinterol ($pEC_{50} = 9.74 \pm 0.15$) increased GT as did insulin ($pEC_{50} = 6.93 \pm 0.15$). The highly selective β_3-AR agonist CL316243 only weakly stimulated GT.

3 The pK_B values calculated from the shift of the pEC_{50} values of the agonists in the presence of the β_1-AR selective antagonist CGP 20712A or the β_3-AR selective antagonist SR 59230A were not indicative of activation of β_1- or β_3-ARs. Only (−)-propranolol and the β_2-AR selective antagonist ICI 118551 caused marked rightward shifts of CR curves to isoprenaline ($pK_B = 10.2 \pm 0.2$ and 9.6 ± 0.3), zinterol ($pK_B = 9.0 \pm 0.1$ and 9.4 ± 0.3) and BRL 37344 ($pK_B = 9.4 \pm 0.3$ and $8.4 \pm .2$), indicating participation of β_2-ARs.

4 The pharmacological analysis was supported by reverse transcription and polymerase chain reaction analysis of L6 mRNA, which showed high levels of expression of β_2-AR but not β_1- or β_3-AR in these cells.

5 Forskolin and dibutyryl cyclic AMP produced negligible increases in GT while the phosphatidylinositol-3 kinase inhibitor, wortmannin, significantly decreased both insulin- and zinterol-stimulated GT, suggesting a possible interaction between the insulin and β_2-AR pathways.

6 This study demonstrates that β_2-ARs mediate the increase in GT in L6 cells to β-AR agonists, including the β_3-AR selective agonist BRL 37344. This effect does not appear to be directly related to increases in cyclic AMP but requires P13K.

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Abbreviations: [^{3}H]-2-DG, 2-deoxy-[^{3}H]-D-glucose; ATCC, American Type Culture Collection; CR, concentration-response curve; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; G_i proteins, inhibitory guanosine triphosphatases; GLUT, glucose transporter; G_S proteins, stimulatory guanosine triphosphatases; GT, glucose transport; HBS, HEPES buffered saline; pEC_{50}, negative log EC_{50}; P13K, phosphatidyl inositol-3 kinase; pK_B, negative log K_B; RT, reverse transcription; WAT, white adipose tissue

Introduction

Insulin-mediated glucose transport (GT) is severely impaired in type 2 diabetes. Since skeletal muscle accounts for ~85% of the total glucose metabolised (DeFronzo *et al*., 1981), there is great interest in identifying insulin-independent mechanisms that stimulate GT in skeletal muscle. Skeletal muscle expresses both GLUT1 and GLUT4 glucose transporters (Gaster *et al*., 2000) and although there is no significant change in GLUT4 protein levels in the skeletal muscle of patients with type 2 diabetes, sensitivity to insulin is reduced in this tissue (Schalin-Jantti *et al*., 1994). In rat tissues *in vivo* the β_3-AR selective agonist BRL 37344 promotes GT (Abe *et al*., 1993; Liu & Stock, 1995), and another selective agonist, CL 316243, decreases blood glucose in obese diabetic mice (Yoshida *et al*., 1994). These effects are not indirectly mediated by an increase in plasma insulin levels, as BRL 37344 and CL 316243 also increase GT in rat isolated tissues, including skeletal muscle, by a mechanism independent of insulin (Abe *et al*., 1993; Liu *et al*., 1996). In the whole muscle preparation, however, these agonists may act on a variety of different cell populations, including adipocytes, which express β_3-AR and respond to β_3-AR agonists with increased GT (Nikami *et al*., 1996). BRL 37344 has also been shown to produce an increase in GT in the absence of insulin in a homogenous population of differentiated L6 rat skeletal muscle cells (Tanishita *et al*., 1997). However, a definitive pharmacological characterisation of the receptor subtype mediating this response has not been made.

Rat skeletal muscle expresses high levels of β_2-AR and moderate levels of β_1- but not β_3-AR mRNA (Roberts *et al*., 1999), and in accord with this finding cyclic AMP levels in rat

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soleus muscle are increased in response to β_2, but not β_3-AR stimulation (Roberts & Summers, 1998). A study that used monoclonal antibodies against human β_3-AR indicated that this receptor was present in human gastrocnemius muscle (Chamberlain *et al*., 1999), although, as in the rat, functional studies in human skeletal muscle have demonstrated the presence of β_2-, but not β_1- or β_3-ARs (Hagström-Toft *et al*., 1998).

L6 cells grow as myoblasts in medium containing 10% foetal calf serum (FBS) and differentiate and undergo fusion into myotubes when transferred to medium containing 2% FBS (Ewart *et al*., 1998). The expression of GLUT1 and GLUT4 glucose transporters in L6 cells is regulated during differentiation (Mitsumoto *et al*., 1991). Since these cells also show an increase in GT in response to BRL 37344 (Tanishita *et al*., 1997) they represent a good model for studying the non-insulin dependent GT mechanisms of skeletal muscle.

The present study aimed to characterize the receptor that mediates GT increase in L6 cells in response to β-AR agonists. We used differentiated L6 myotubes to measure GT using the 2-deoxy-[^{3}H]-D-glucose ([^{3}H]-2-DG) method (Tanishita *et al*., 1997) and a number of selective agonists and antagonists to characterize the β-AR involved.

Methods

Cell culture

Rat L6 myoblasts (ATCC) were grown as a monolayer in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g l^{-1} glucose, supplemented with 10% FBS, 8 mM L-Glutamine, 10 mM HEPES buffer, 2.5 μg ml^{-1} fungizone (Amphostat B) and 80 μg ml^{-1} gentamycin sulphate, at 37°C in air containing 5% CO_2. For differentiation, the cells were seeded into 12-well plates at $\sim 8 \times 10^5$ cells per well and maintained in DMEM supplemented with 2% FBS for 7 days until fusion occurred, with medium renewal every second day. The cells used for [^{3}H]-2-DG assays and reverse transcription/polymerase chain reaction (RT/PCR) experiments were restricted to passages 5 to 10.

2-deoxy-[^{3}H]-D-glucose uptake assay

GT was measured using the [^{3}H]-2-DG glucose method (Tanishita *et al*., 1997). Briefly, the cells were washed with HEPES Buffered Saline (HBS) and incubated in serum free DMEM for 16–20 h the day before each experiment. [^{3}H]-2-DG transport was measured on day 7. Cytochalasin B (10 μM) was used to determine non-facilitated GT (Birnbaum, 1989). In our experiments cytochalasin B inhibited the basal GT by 70–75%. [^{3}H]-2DG (50 nM) uptake was allowed to proceed for 15 min at 37°C, and then terminated by rapid washing with ice-cold HBS. The uptake of [^{3}H]-2DG during this time was linear. For quantitation of [^{3}H]-2-DG uptake cells were digested with 0.2 M NaOH for 2–3 h at 70°C, mixed with 3.5 ml EcoLite and counted.

RNA extraction

RNA was extracted from L6 cells at day 7, and from heart, cerebellum and white adipose tissue (WAT) of a male Sprague Dawley rat (280 g) using Trizol reagent according to the manufacturer's protocol (Life Technologies, Inc.). The RNA was assessed for purity by 260/280 absorbency ratio and gel electrophoresis. All RNA samples were frozen at −70°C, until they were used for RT/PCR within 2–3 months.

Reverse transcription (RT) and polymerase chain reaction (PCR)

RT was performed on 1 μg of isolated RNA, using RT reaction mix consisting of 10 × RT buffer (Promega), 10 mM dNTPs, 0.5 μg μl^{-1} oligo(dT), 20 mM $MgCl_2$, and 20 U reverse transcriptase and 18 U RNAsin (Promega). The samples were incubated at 42°C for 45 min, then at 95°C for 5 min before being placed on ice. 20 μl of 1 mM EDTA was added to each sample, and the RT products were then stored at −70°C until they were used for PCR within 1 month. PCR amplification was performed on 4 μl of cDNA. Specific oligonucleotide rat primers were used to amplify β_1-AR (forward, 5′-CCGCTGCTACAACGACCCCAAG-3′ and reverse, 5′-AGCCAGTTGAAGAAGACGAAGAGGCG-3′), β_2-AR (forward, 5′-GGTTATCGTCCTGGCCATCGTGTTTG-3′ and reverse, 5′-TGGTTCGTGAAGAAGTCACAGCAAGTCTC-3′), β_3-AR (intron spanning forward, 5′-TAGTCCTGGTGTGGATCGTGTCCGC-3′ and reverse, 5′-CGCTCACCTTCATAGCCATCAAACC-3′), and β-actin (forward, 5′-ATCCTGCGTCTGGACCTGGCTG-3′ and reverse, 5′-CCTGCTTGCTGATCCACATCTGCTG-3′). One of the primers for each PCR reaction was labelled with γ^{33}P-adenosine 5′-triphosphate (Geneworks). PCR reactions contained 1 × PCR buffer (Life Technologies), 200 μM dNTP's, 2 mM magnesium acetate, 40 ng of each primer and 1 U Taq polymerase (Life Technologies), and then amplified for 16 (β-actin) and 27 (β_2- and β_3-ARs) cycles at 64°C, or 30 cycles at 60°C (β_1-AR). Following amplification, 4 μl of PCR products were electrophoresed on a 1.3% agarose gel. The gel was then transferred onto Hybond N+ membrane by Southern blotting in 0.4 M NaOH/1 M NaCl overnight. After blotting, the membrane was soaked in 0.5 M Tris-HCl (pH 7.5)/1 M NaCl for 5 min, washed in 2 × SSC (0.3 M NaCl/30 mM sodium citrate) for 5 min, and air-dried for 30 min. The membranes were exposed to phosphorimager plates for 6–20 h and the bands quantified using the ImageQuaNT software (Molecular Dynamics). The β-actin was used as an internal control.

Drugs and reagents

The authors would like to thank the following companies and individuals for gifts of: SR 59230A, 3-(2-ethylphenoxy)-1-[(1S)-1,2,3,4-tetrahydronapth-1-ylamino]-2S-2-propranol oxalate (Dr L. Manara, Sanofi-Midi), CL 316243, (R,R)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino]-propyl]1,3-benzodioxole-2,2-dicarboxylate (Dr T. Nash, Wyeth-Ayerst) and (±)-CGP 20712A (2-hydroxy-5(2-((2-hydroxy-3-(4-((1-methyl-4-trifluoromethyl)1H-imidazole-2-y1)-phenoxy)propyl)amino)ethoxy)-benzamide monomethane sulphonate) (Dr G. Anderson, Ciba-Geigy AG Switzerland). The drugs and reagents used were as follows: zinterol hydrochloride (Bristol-Myers Squibb, Noble Park, Australia); (−)-propranolol, (±)-ICI 118551, (erythro-DL-1(7-methylin-dian-4-yloxy)-3-

isopropylaminobutan-2-ol) (Imperial Chemical Industries Wilmslow, Cheshire, U.K.); BRL 37344, 4-[2-[(2-hydroxy-2-(3-chlorophenyl)ethyl)-amino]propyl]-phenoxyacetic acid (Tocris Cookson Ltd., Ballwin MO, U.S.A.); (−)-isoprenaline, wortmannin, forskolin, dibutyryl cyclic AMP (N^6,2′-O-dibutyryladenosine 3′.5′-cyclic monophosphate), cytochalasin B, insulin (Sigma Chemical Co., St. Louis MO, U.S.A.); DMEM, L-glutamine, Amphostat B, HEPES buffer, trypsin/EDTA (Trace Biosciences, Sydney, Australia); gentamicin sulphate (David Bull Laboratories, Melbourne, Australia); 2-deoxy-[^{3}H]-D-Glucose (NEN™ Life Science Products, Inc., Boston MA, U.S.A.); Trizol reagent (Life Technologies, Rockville MD, U.S.A.). Stock solutions of forskolin, cytochalasin B and wortmannin were made up in 10% dimethyl sulphoxide; the final concentration of dimethyl sulphoxide in the assay media was 0.01%. The remaining drugs were made up in 2 mM L(+)-ascorbic acid. None of the vehicles had statistically significant effects on the basal GT. The primers were synthesised at: Gibco BRL, Life Technologies, Rockville MD, U.S.A., Horward Florey Institute, Melbourne, Australia or Gene Works Pty Ltd., Australia.

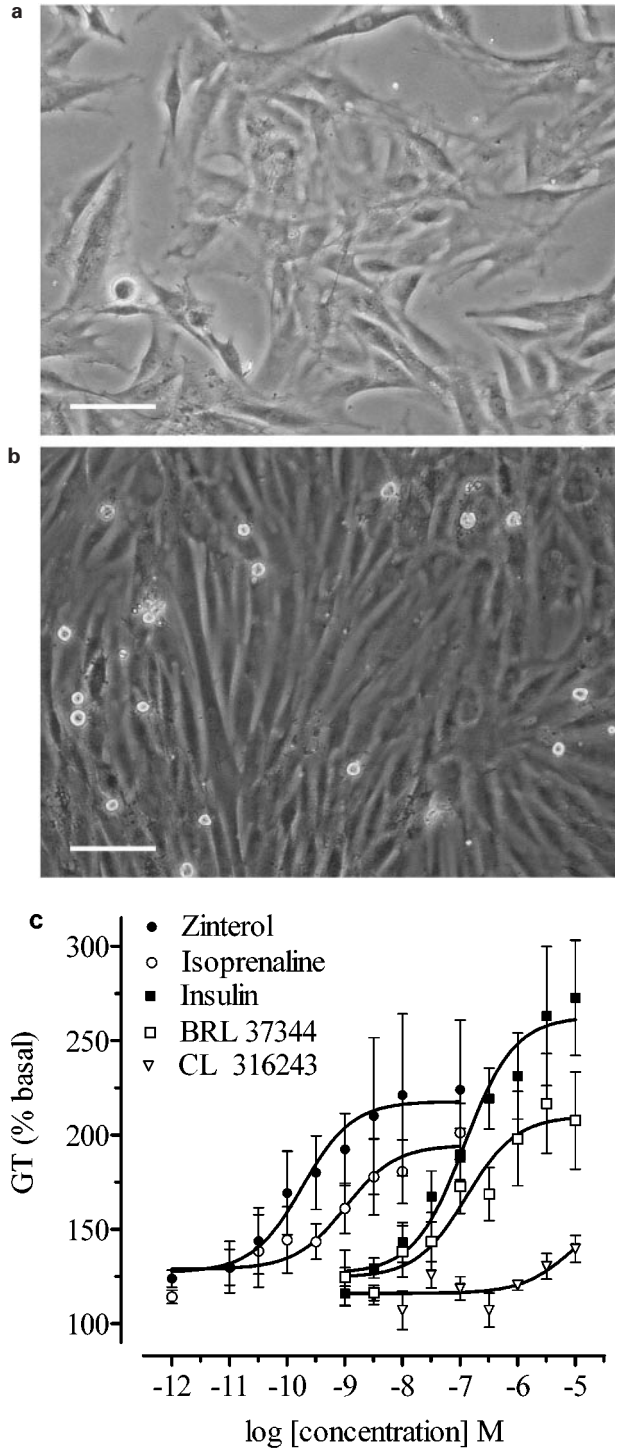

Figure 1 (a) Undifferentiated L6 cells. Scale bar = 45 μm. (b) L6 myotubes differentiated for 7 days. (c) CR curves for accumulation of [^{3}H]-2-DG were constructed for insulin, BRL 37344, CL 316243, zinterol and isoprenaline, in L6 myotubes. The graph shows mean ± s.e.mean, $n = 6-10$.

Statistics

Results are presented as mean values ± s.e.mean. The statistical significance of differences between the control (untreated cells) and experimental groups was analysed by Student's unpaired *t*-test, one-way ANOVA or two-way ANOVA followed when appropriate by Student's unpaired *t*-test at the levels indicated.

Results

The effects of insulin and β-AR agonists on glucose transport in L6 myotubes

Insulin-stimulated glucose transport (GT) was used in most experiments as a positive control for cell viability. Concentration-response (CR) curves for stimulation of GT were constructed using L6 cells differentiated into myotubes (Figure 1a,b). GT was stimulated by insulin, the non-selective β-AR agonist isoprenaline, the β_2-AR agonist zinterol, and the β_3-AR agonist BRL 37344 (Table 1 and Figure 1c). The magnitude of the BRL 37344-induced increase in GT was consistent with previous findings (Tanishita *et al*., 1997). BRL 37344 and insulin displayed similar pEC_{50} values, whereas isoprenaline and zinterol had potencies about three orders of

Table 1 Effects of the β-AR agonists on [^{3}H]-2DG transport in L6 cells

Treatment (n)	*pEC_{50}* (means ± s.e.)	*Maximal increase in GT* (% GT ± s.e.)
Insulin (9)	6.93 ± 0.15	273 ± 30
BRL 37344 (10)	6.89 ± 0.21	217 ± 26
CL 316243 (6)	NA	140 ± 7
Isoprenaline (7)	8.99 ± 0.24	201 ± 15
Zinterol (6)	9.74 ± 0.15	224 ± 36

The table shows the pEC_{50} values and the maximal responses to insulin and β-AR agonists, expressed as mean ± s.e. of n experiments performed in duplicates. The maximal responses were expressed as per cent of the basal GT.

magnitude greater. The β-AR agonists produced similar maximal responses to insulin. A highly selective β_3-AR agonist CL 316243 (Yoshida *et al*., 1994) induced a small increase in GT, but only at the highest concentration used (Table 1, Figure 1c).

The effect of antagonists on β-AR stimulated glucose transport

To characterize the receptor subtype stimulated by the β-AR agonists, antagonists selective for each receptor subtype (β_1-,

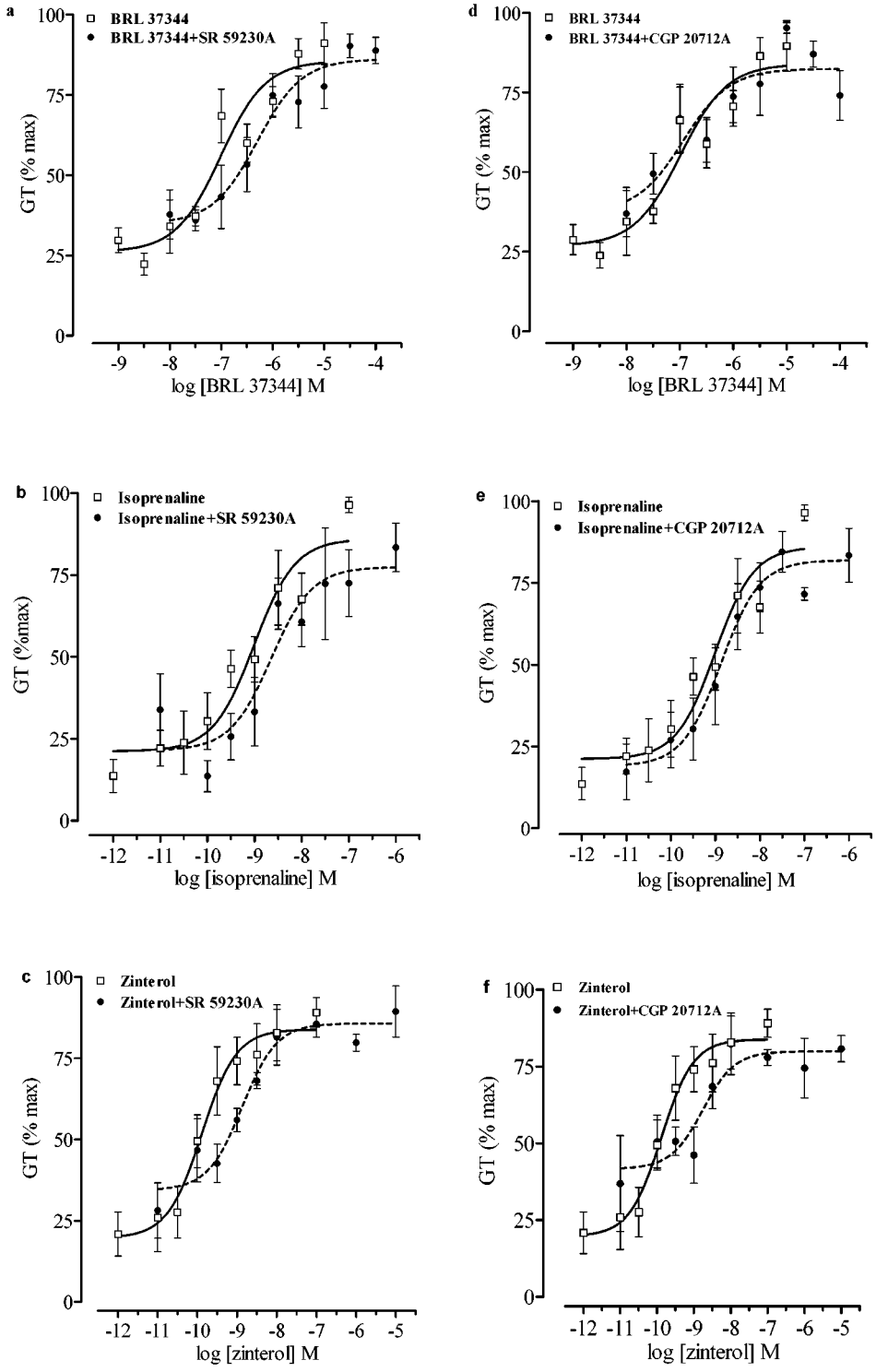

Figure 2 [^{3}H]-2-DG transport in response to BRL 37344, zinterol and isoprenaline in the absence or presence of 10^{-7} M of the β_3-AR selective antagonist SR 59230A (a, b and c), or the β_1-AR selective antagonist CGP 20712A (d, e and f) in L6 myotubes. The graphs show mean ± s.e. of the mean, $n = 4-5$.

β_2- and β_3-ARs) were employed. Analysis of the data by two-way ANOVA ($P<0.05$) showed that the β_3-AR selective antagonist SR 59230A and the β_1-AR selective antagonist CGP 20712A caused significant dextral shifts in the CR curves to zinterol and isoprenaline (Figure 2b,c,e,f), but not BRL37344 (Figure 2a,d). However, application of Student's *t*-test showed that the differences in the pEC_{50} values for isoprenaline in the presence and absence of SR59230A or CGP20712A were not statistically significant. Since dextral shifts in the CR curve to zinterol in the presence of SR59230A and CGP20712A were not observed in all individual experiments, pK_B values for the antagonists were calculated from the mean pEC_{50} values of zinterol. The pK_B values calculated for CGP20712A (5.9) and SR59230A (6.0) were not appropriate for an action at β_1- or β_3-ARs respectively (Table 2). In contrast, the β_2-AR selective antagonist ICI 118551 caused marked and significant dextral shifts in the CR curves to BRL 37344, isoprenaline and zinterol (Figure 3a–c). The non-selective β-AR antagonist propranolol was used to further assess the receptor subtype stimulated by these agonists. In the presence of propranolol significant shifts to the right in the CR curves to all three β-AR agonists were observed (Table 2, Figure 3d–f). The pK_B values calculated for ICI118551 and propranolol using all three agonists were appropriate for an action at β_2-ARs.

The effects of β-AR antagonists on insulin-stimulated glucose transport

To test whether CGP 20712A, ICI 118551, SR 59230A or propranolol have any effect on GT other than by their effects on the β_2-AR, L6 cells were treated with insulin in the presence or absence of these antagonists (10^{-7} M). Analysis using two-way ANOVA showed that the response to insulin was significantly affected by the presence of SR59230A ($P<0.05$). However, application of Student's *t*-test demonstrated no significant difference between the pEC_{50} values or the maximal responses to insulin in the presence or absence of any of the four antagonists (Table 3).

Measurement of β-AR subtype mRNA expression by RT/PCR

RT/PCR experiments were conducted to examine whether β_1, β_2 or β_3-ARs are expressed in L6 cells differentiated for 7 days. β_2-AR mRNA was expressed at high levels, whereas β_3-AR mRNA was not expressed at all, and β_1-AR mRNA showed negligible expression (Figure 4). Positive controls for all three β-AR subtypes were included in the RT/PCR experiment. Readily detectable β_3-AR mRNA in rat WAT and β_1-AR mRNA in rat heart indicates that the absence or low expression levels of β_1- and β_3-AR mRNA in L6 cells was not due to a failure of the RT/PCR reaction.

The effect of forskolin and dibutyryl cyclic AMP on glucose transport

Further experiments were carried out to elucidate the possible mechanisms by which BRL 37344, isoprenaline and zinterol stimulated GT in L6 cells. The classical pathway stimulated by β_2-AR activates adenylate cyclase and increases cellular cyclic AMP levels. Forskolin directly stimulates adenylate cyclase and increases production of cyclic AMP, whereas dibutyryl cyclic AMP is a cell-permeable analogue of cyclic AMP. CR curves to forskolin and dibutyryl cyclic AMP were constructed to examine whether cyclic AMP is involved in stimulation of GT in L6 cells (Figure 5c). Although GT did appear to be increased by both forskolin ($141\pm13\%$ at 10^{-6} M, $n=4$) and dibutyryl cyclic AMP ($144\pm8\%$ at 10^{-5} M, $n=5$), comparison of the responses with the basal rate of GT showed that the increases were not statistically significant (one-way ANOVA, $P>0.05$), suggesting that the ability of forskolin to increase cyclic AMP levels or the direct application of a cyclic AMP analogue may not be directly coupled to facilitation of GT.

The effect of wortmannin on insulin- and zinterol-stimulated glucose transport

Wortmannin is an inhibitor of phosphatydylinositol-3 kinase (PI3K) and inhibits insulin stimulated GT and GLUT translocation to the plasma membrane (Srivastava, 1998). CR curves to insulin and zinterol were constructed in the absence or presence of wortmannin to test whether β-AR agonists cause an increase in GT *via* a pathway similar to that of insulin (Figure 5a,b). The maximal responses to insulin (10^{-5} M, $236\pm20\%$) and zinterol (10^{-7} M, $200\pm25\%$) without wortmannin were compared with the responses in the presence of wortmannin (insulin, $120\pm17\%$; zinterol, $142\pm10\%$). Inhibition of responses to insulin and zinterol by wortmannin were statistically significant (insulin, $n=7$, $P<0.001$; zinterol, $n=7$, $P<0.05$). The basal level of GT was not affected by wortmannin ($P=0.9$, $n=6$).

The effect of combining insulin and the β-AR agonists on glucose transport

To examine the possible interaction between the insulin and β-AR agonist pathways, L6 cells were treated with the

Table 2 Effects of β-AR agonists on the CR curves for stimulation [^{3}H]-2-DG transport in L6 cells by β-AR agonists

	BRL 37344		*Isoprenaline*		*Zinterol*	
	pEC_{50} (mean±s.e.)	pK_B	pEC_{50} (mean±s.e.)	pK_B	pEC_{50} (mean±s.e.)	pK_B
CGP 20712A	6.98±0.25	NS	9.01±0.23	NS	9.89±0.11	5.9*
ICI 118551	7.04±0.23	8.4±0.2***	9.12±0.21	9.6±0.3***	9.79±0.16	9.4±0.3***
SR 59230A	7.01±0.25	NS	9.01±0.23	NS	9.89±0.11	6.0**
Propranolol	7.51±0.18	9.4±0.3***	9.28±0.14	10.2±0.2***	9.81±0.19	9.0±0.1***

Data are mean±s.e. of 4–6 individual experiments performed in duplicate. The cells were treated with BRL 37344, zinterol or isoprenaline in the absence or presence of 10^{-7} M of CGP 20712A, ICI 118551, SR 59230A or propranolol. The pK_B values were calculated from the pEC_{50} values of the agonists. ***$P<0.001$, **$P<0.01$, *$P<0.05$, NS – not significant.

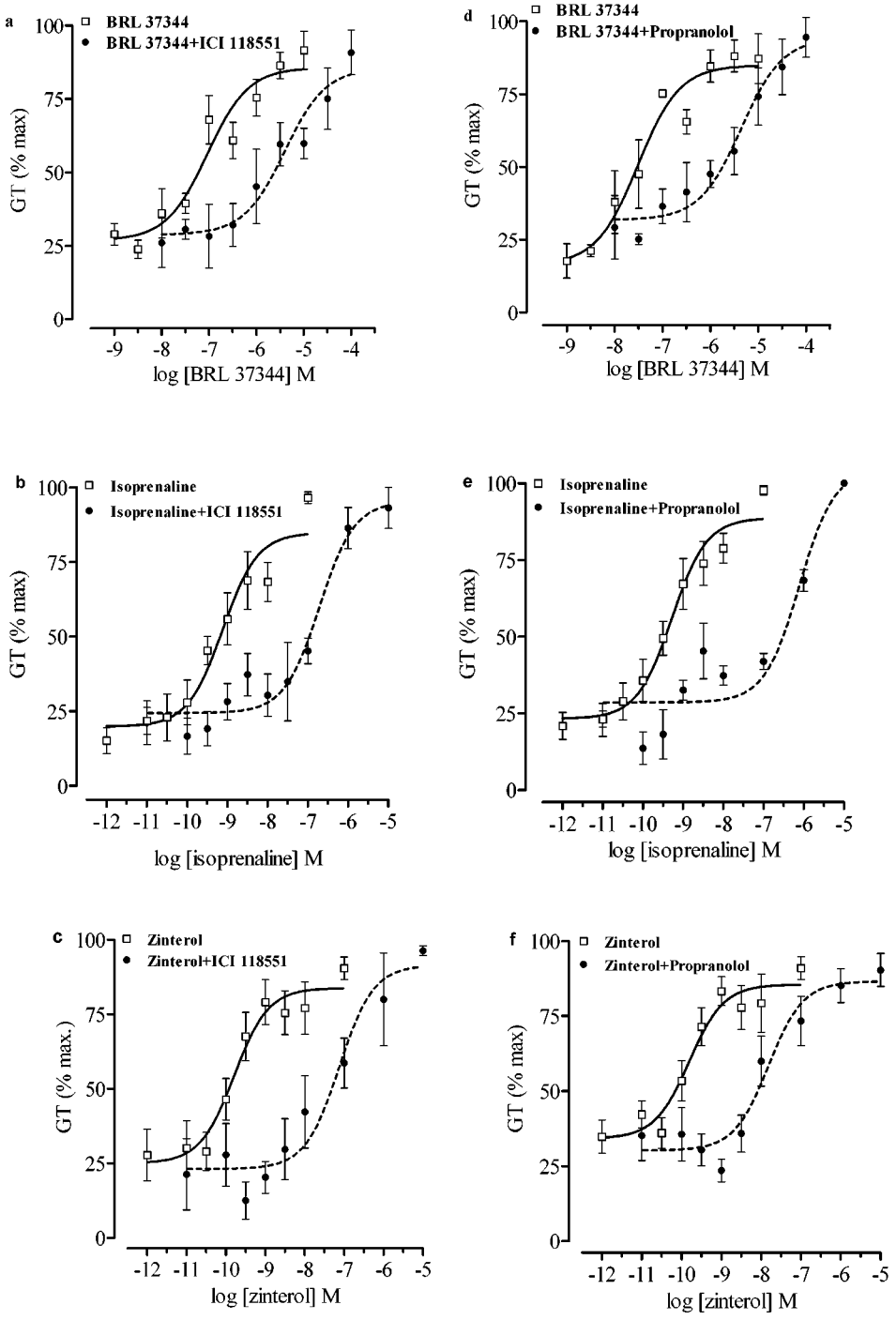

Figure 3 [^{3}H]-2-DG transport in response to BRL 37344, zinterol and isoprenaline in the absence or presence of 10^{-7} M of the β_2-AR selective antagonist ICI 118551 (a, b and c) or the non-selective β-AR antagonist propranolol (d, e, and f) in L6 myotubes. The graphs show mean$\pm$s.e.mean, $n=4-6$.

agonists in the absence or presence of insulin at 10^{-5} M. In the presence of insulin the responses to BRL 37344, isoprenaline and zinterol were significantly potentiated (Table 4 and Figure 6).

Discussion

The present study provides strong evidence that the increase in GT stimulated by the β_3-AR agonist BRL 37344 in L6 cells is mediated *via* β_2-AR. Although it is known that BRL37344 can activate β_2-AR (Board *et al*., 2000) several previous reports have shown that BRL 37344 stimulates GT in rat isolated skeletal muscle and in the rat skeletal muscle cell line L6 and suggested that the effect was mediated by atypical β-AR or β_3-AR (Liu *et al*., 1996; Tanishita *et al*., 1997). GT in differentiated L6 myotubes was studied using the selective β_3-AR agonists, BRL 37344 and CL 316243, the selective β_2-AR agonist zinterol, and the non subtype-

Table 3 Effects of β-AR antagonists on the insulin stimulated [^{3}H]-2-DG transport in L6 cells

	pEC50 (mean ± s.e.)			*Insulin % GT* (mean ± s.e.)		
	Without antagonist	*With antagonist*	P *(n)*	*Without antagonist*	*With antagonist*	P *(n)*
CGP 20712A	7.03 ± 0.09	7.02 ± 0.16	1.0 (4)	276 ± 44	234 ± 40	0.5 (4)
ICI 118551	6.88 ± 0.21	7.17 ± 0.23	0.4 (4)	301 ± 49	249 ± 32	0.4 (4)
SR 59230A	6.72 ± 0.21	6.98 ± 0.25	0.5 (3)	308 ± 58	313 ± 59	1.0 (3)
Propranolol	6.95 ± 0.18	7.22 ± 0.24	0.4 (4)	295 ± 52	278 ± 31	0.8 (4)

L6 cells were incubated in the absence or presence of 10^{-7} M of CGP 20712A, ICI 118551, SR 59230A or propranolol. The values are mean ± s.e. of 3–4 individual experiments performed in duplicate.

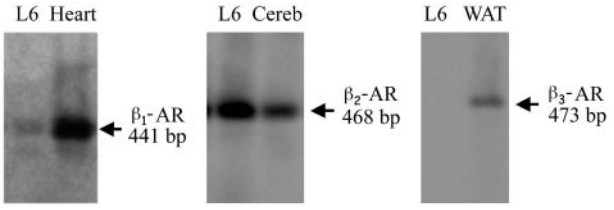

Figure 4 The figure shows β_1- (441 bp), β_2- (468 bp) and β_3-AR (473 bp) PCR products from rat heart, cerebellum and WAT, and β_2-AR PCR product from L6 myotubes. Levels of β_1-, β_2- and β_3-ARs expression were measured using RT/PCR analysis.

selective β-AR agonist isoprenaline. Selective antagonists for each β-AR subtype and the non-selective β-AR antagonist propranolol were employed to characterise the receptor subtype mediating increase in GT in these cells.

Although BRL 37344 stimulated GT in the absence of insulin in accord with the previous findings (Tanishita *et al*., 1997), another potent and highly selective β_3-AR agonist CL 316243 had only a very weak effect on GT in L6 cells. Previous studies have shown that CL 316243 is some 10,000 fold selective for β_3-ARs vs β_1- and β_2-AR, is active in the nanomolar range (Dolan *et al*., 1994), and is ineffective in β_3-AR knockout animals (Cohen *et al*., 1999). Both the β_2-AR selective agonist zinterol and non-selective agonist isoprenaline were much more potent than BRL 37344 in stimulating GT. The selective β_2-AR antagonist ICI 118551 and non-selective β-AR antagonist propranolol were highly effective in inhibiting BRL 37344, zinterol and isoprenaline stimulated GT. The calculated pK_B values for both the antagonists were as expected for β_2-AR blockade (Roberts & Summers, 1998) against all of the agonists. Molecular analysis of β_2-AR mRNA expression revealed that β_2-AR but not β_1- or β_3-AR mRNA is expressed at high levels in L6 cells as in a previous study in rat soleus muscle (Roberts *et al*., 1999), and in a more recent study in L6 cells (Nagase *et al*., 2001). The β_3-AR selective antagonist SR 59230A failed to inhibit the response to the non-selective β-AR agonist, isoprenaline, indicating that in these cells, isoprenaline did not act at β_3-ARs. SR 59230A caused a small shift in the CR curve to the β_2-AR selective agonist zinterol, but the shift was not consistent. The pK_B value for this antagonist calculated from the mean pEC_{50} values of zinterol was much lower (6.0) than the pA_2 value for SR59230A at the β_3-AR which has been reported as 8.76 (Manara *et al*., 1996), indicating that the agonist was acting at β_2- and not β_3-ARs. Stimulation of GT by β-AR agonists did not involve β_1-AR since the β_1-AR selective antagonist CGP 20712A failed to block the responses to BRL 37344 and isoprenaline. Although there was a small shift in the CR curve to zinterol it was not consistent for all individual experiments, and the pK_B value was much lower (5.9) than expected for the action at the β_1-ARs. If zinterol were acting at β_1-ARs in L6 cells, CGP 20712A at 10^{-7} M would have a pK_B value close to 9.6 (Kaumann, 1997). These results suggest that GT stimulated by BRL 37344, isoprenaline and zinterol is mediated neither by β_1- nor by β_3-ARs. Rather, the high potency of zinterol and isoprenaline, blockade by ICI 118551 and propranolol, presence of high levels of β_2-AR mRNA together with the poor or non-existent antagonism by β_1- or β_3-AR antagonists and lack of expression of β_1- or β_3-AR mRNA provide strong indications that GT responses in L6 cells to BRL 37344, isoprenaline and zinterol are mediated entirely by β_2-ARs.

In all systems examined, including soleus muscle (Roberts & Summers, 1998), β_2-ARs couple to the stimulatory G protein (G_s), which activates adenylate cyclase and causes an increase in intracellular cyclic AMP. It was therefore important to establish whether stimulation of GT by β_2-ARs could be mimicked by increased cyclic AMP levels in L6 cells. GT was not significantly increased by high concentrations of the direct adenylate cyclase stimulant forskolin, or the cell permeable analogue dibutyryl cyclic AMP, suggesting that cyclic AMP may have little direct role in stimulation of GT in L6 cells. However, it should be borne in mind that forskolin has been shown to inhibit GT in cultured fibroblast cells (Tokuda *et al*., 1994), possibly through a direct action on glucose transporters (Sergeant & Kim, 1985; Klip *et al*., 1988). It has also been shown that treatment of placental cells with an analogue of cyclic AMP (8-bromo-cyclic AMP), an activator of G_s protein (cholera toxin), or forskolin results in inhibition of GT (Sakata *et al*., 1996), which suggests that elevation of cyclic AMP can inhibit GT. In rat soleus muscle, zinterol and isoprenaline, but not BRL37344, cause accumulation of cyclic AMP (Roberts & Summers, 1998), and all three agonists increase cyclic AMP in L6 cells (Nevzorova *et al*., unpublished observations). BRL37344 thus increases GT in both rat soleus muscle (Abe *et al*., 1993; Liu *et al*., 1996) and L6 cells (Tanashita *et al*., 1997 and present results) yet increased cyclic AMP was observed only in L6 cells. This lack of correlation between cyclic AMP levels and GT suggests that β_2-AR might also couple to a pathway not involving cyclic AMP, and further experiments employed an inhibitor of PI3K, wortmannin, to determine if this pathway was utilised by zinterol to increase GT in L6 cells.

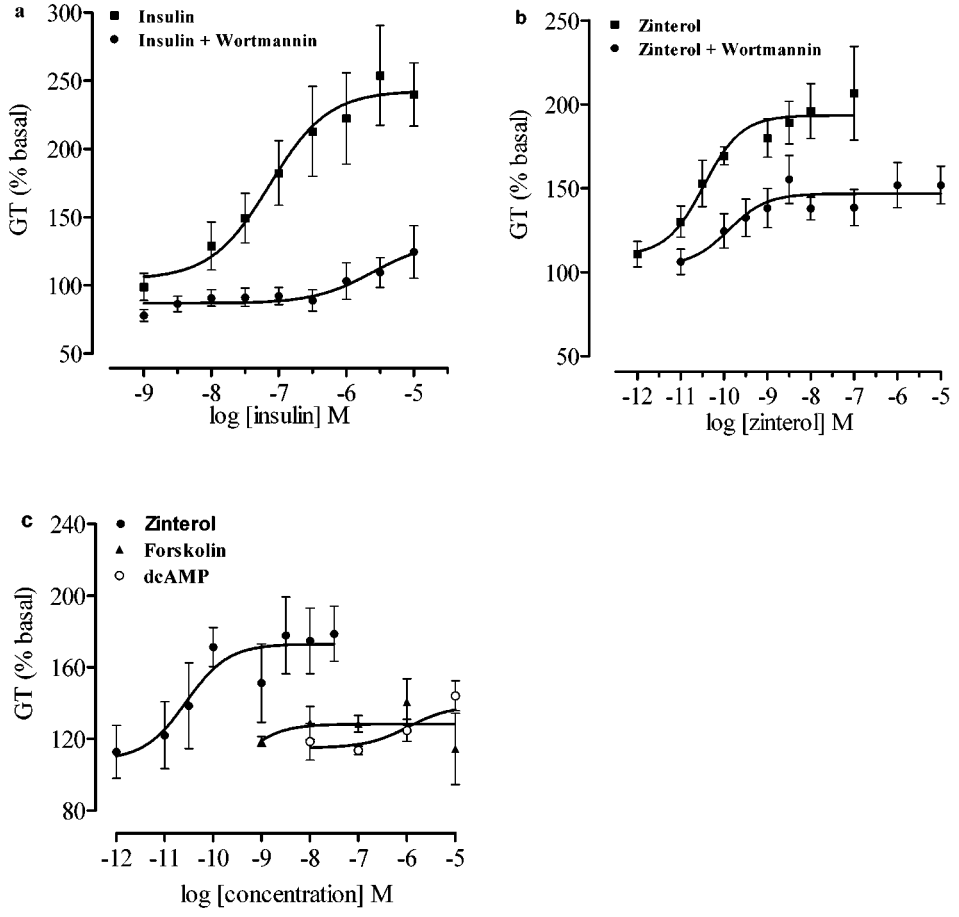

Figure 5 (a,b) [^{3}H]-2-DG transport in response to insulin and zinterol in the absence or presence of PI3K inhibitor wortmannin (10^{-7} M) in L6 myotubes. (c) CR curves for accumulation of [^{3}H]-2-DG by forskolin and dibutyryl cyclic AMP in L6 myotubes. The graphs show mean±s.e.mean, $n=4-6$.

Table 4 Effects of insulin on the β-AR stimulated [^{3}H]-2DG transport in L6 cells

(n)	% GT (mean±s.e.) without insulin	% GT (mean±s.e.) with insulin	P
BRL 37344 (5)	166±12	282±14	$P<0.001$
Isoprenaline (4)	183±4	327±11	$P<0.0001$
Zinterol (5)	202±8	336±18	$P<0.001$
Insulin (5)	244±15	–	NA

L6 cells were incubated with the β-AR agonists (10^{-5} M) in the absence or presence of insulin (10^{-5} M). The increase in GT was expressed as per cent of the basal GT, mean±s.e. Statistical significance was tested between the β-AR agonists alone and β-AR agonists+insulin groups.

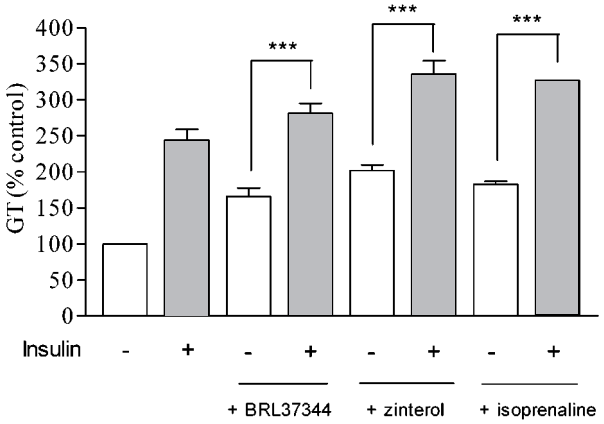

Figure 6 [^{3}H]-2-DG transport in response to 10^{-5} M of BRL 37344, zinterol and isoprenaline in the absence or presence of insulin (10^{-5} M). The histograms are mean±s.e.mean, $n=4-5$.

Wortmannin inhibits insulin-stimulated GT and translocation of glucose transporters to the plasma membrane (Shepherd *et al*., 1997; Tsakiridis *et al*., 1995). In the present study, wortmannin inhibited GT stimulated by both insulin and zinterol. Similar effects on insulin-stimulated GT are observed with another inhibitor of PI3K, LY 294002 (Srivastava, 1998), and preliminary experiments in our laboratory show that LY 294002 inhibits both insulin and zinterol stimulated GT in L6 cells (data not shown). In contrast, BRL 37344-stimulated GT in L6 cells was reported to be unaffected by wortmannin (Tanishita *et al*., 1997). However, as this study used a single concentration of BRL 37344, it was not possible to determine whether wortmannin produced a shift in the CR curve to BRL 37344. Our findings suggest that the pathway downstream of PI3K may be

common for insulin and β_2-ARs. There is evidence that the β_2-AR can form signalling complexes with receptor tyrosine kinases, including the epidermal growth factor receptor (Maudsley *et al*., 2000), and the insulin receptor (Wang *et al*., 2000). In addition, the insulin and insulin-like growth factor-1 receptors have been shown to interact with components of G-proteins (Dalle *et al*., 2001). Thus there is a possibility that the β_2-AR can directly interact with the insulin receptor in the stimulation of GT. There is some evidence that β-arrestins, that couple to phosphorylated β-AR, can act as adaptors between G-protein coupled receptors and other signalling pathways (Miller & Lefkowitz, 2001).

To further examine whether insulin and β-AR agonists stimulated GT in L6 cells through distinct mechanisms, cells were incubated with supramaximal concentrations of the β-AR agonists alone or in the presence of insulin. The results show that the addition of insulin significantly increases responses to BRL 37344, isoprenaline and zinterol compared to responses to the agonists alone. However, the responses were not additive probably indicating that insulin and zinterol utilise two pathways which overlap, probably at the level of PI3K.

In conclusion, the present study demonstrates that the selective β_3-AR agonist, BRL 37344, the β_2-AR agonist, zinterol, and the non-selective β-AR agonist, isoprenaline, all stimulate GT in differentiated rat skeletal muscle L6 cells via β_2-ARs. Molecular analysis of β-AR expression in L6 cells provided further support for these findings, demonstrating that only β_2-AR mRNA is highly expressed in these cells. The present study contrasts with previous findings that suggest that stimulation of GT in L6 cells by the β_3-AR agonist, BRL 37344, is mediated *via* atypical or β_3-ARs. The experiments with forskolin and dibutyryl cyclic AMP to investigate the role of cyclic AMP in GT were inconclusive and the role of cyclic AMP needs to be investigated further. In addition, the use of PI3K inhibitor, wortmannin, suggested that the mechanisms of both insulin and β_2-AR stimulated GT in L6 cells involve PI3K. Further characterization of β_2-AR mediated GT will assist in understanding the mechanisms involved.

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