British Journal of Pharmacology (2008) 155, 395–406
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0007–1188/08 $32.00
www.brjpharmacol.org

RESEARCH PAPER

Metabolic responses to BRL37344 and clenbuterol in soleus muscle and C2C12 cells via different atypical pharmacologies and β_2-adrenoceptor mechanisms

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Background and purpose: Picomolar concentrations of the β_3-adrenoceptor agonist BRL37344 stimulate 2-deoxyglucose uptake in soleus muscle via undefined receptors. Higher concentrations alter uptake, apparently via β_2-adrenoceptors. Effects of BRL37344 and β_2-adrenoceptor agonists are compared.
Experimental approach: Mouse soleus muscles were incubated with 2-deoxy[1-^{14}C]-glucose, [1-^{14}C]-palmitate or [2-^{14}C]-pyruvate, and BRL37344, β_2-adrenoceptor agonists and selective β-adrenoceptor antagonists. Formation of 2-deoxy[1-^{14}C]-glucose-6-phosphate or $^{14}CO_2$ was measured. 2-Deoxy[1-^{14}C]-glucose uptake and β-adrenoceptor mRNA were measured in C2C12 cells.
Key results: 10 pM BRL37344, 10 pM clenbuterol and 100 pM salbutamol stimulated 2-deoxyglucose uptake in soleus muscle by 33–54%. The effect of BRL37344 was prevented by 1 μM atenolol but not by 300 nM CGP20712A or ICI118551, or 1 μM SR59230A; that of clenbuterol was prevented by ICI118551 but not atenolol. 10 nM BRL37344 stimulated 2-deoxyglucose uptake, whereas 100 nM clenbuterol and salbutamol inhibited uptake. These effects were blocked by ICI118551. Similar results were obtained in C2C12 cells, in which only β_2-adrenoceptor mRNA could be detected by RT-PCR. 10 nM BRL37344 and 10 pM clenbuterol stimulated muscle palmitate oxidation. In the presence of palmitate, BRL37344 no longer stimulated 2-deoxyglucose uptake and the effect of clenbuterol was not significant.
Conclusions and implications: Stimulation of glucose uptake by 10 pM BRL37344 and clenbuterol involves different atypical pharmacologies. Nanomolar concentrations of BRL37344 and clenbuterol, probably acting via β_2-adrenoceptors, have opposite effects on glucose uptake. The agonists preferentially stimulate fat rather than carbohydrate oxidation, but stimulation of endogenous fat oxidation cannot explain why 100 nM clenbuterol inhibited 2-deoxyglucose uptake.
British Journal of Pharmacology (2008) **155,** 395–406; doi:10.1038/bjp.2008.244; published online 16 June 2008

Keywords: β-adrenoceptor; atypical β-adrenoceptor; BRL37344; clenbuterol; salbutamol; soleus muscle; glucose uptake; C2C12 cells; fatty acid oxidation; ligand-directed signalling

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; dNTP, deoxynucleoside triphosphate; RT, reverse transcriptase

Introduction

β-Adrenoceptor agonists affect blood glucose homoeostasis in rodents by multiple mechanisms. Repeated administration of β_3-adrenoceptor agonists improves insulin sensitivity in insulin-resistant rodents, perhaps because they increase fatty acid oxidation and lower intracellular lipid metabolite concentrations (Arch, 2002; Darimont *et al.*, 2004). Single doses of β_3-adrenoceptor agonists, in contrast, lower blood glucose in insulin-sensitive but not in insulin-resistant rodents. This effect is a consequence of increased insulin secretion, which is, in turn, probably a consequence of increased lipolysis and stimulation of insulin secretion by non-esterified fatty acids (Grujic *et al.*, 1997). Single doses of β_2-adrenoceptor agonists raise blood glucose levels by stimulating hepatic glucose output (Smith *et al.*, 1990), possibly because they stimulate glucagon secretion (Lacey *et al.*, 1991; Yoshida *et al.*, 1991).

β-Adrenoceptor agonists also affect glucose homoeostasis acutely, through direct effects on both brown adipocytes (Chernogubova *et al.*, 2005) and skeletal muscle—the subject

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Received 12 March 2008; revised 13 May 2008; accepted 20 May 2008; published online 16 June 2008

of the present work. Very low concentrations of the selective β_3-adrenoceptor agonist, BRL37344 (*RR* + *SS*-(±)-4-(2-[(2-(3-chlorophenyl)-2-hydroxyethyl)amino]propyl)phenoxyacetic acid) stimulated 2-deoxyglucose uptake by rat-isolated soleus and extensor digitorum longus muscle *in vitro* (Abe *et al*., 1993; Liu *et al*., 1996a). This effect was not blocked by atenolol (β_1-adrenoceptor antagonist), ICI118551 ((±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl) amino]-2-butanol hydrochloride) (β_2-adrenoceptor antagonist) or (except at a high concentration) by SR59230A (3-(2-ethylphenoxy)-1[(1*s*)-1,2,3,4-tetrahydronaphth-1-ylamino]-2*S*-propanol oxalate) (β_3-adrenoceptor antagonist) (Liu *et al*., 1996a, b). Moreover, BRL37344 had its maximal effect at concentrations (10–100 pM) below its EC_{50} concentration (2 nM) for stimulation of lipolysis via rodent β_3-adrenoceptors and far below concentrations that stimulate responses mediated by rodent β_1- or β_2-adrenoceptors (Arch *et al*., 1984). Very low concentrations (10–100 pM) also stimulated [1-^{14}C]-palmitate and [2-^{14}C]-pyruvate oxidation in soleus muscle from both wild-type (Board *et al*., 2000a) and, according to a preliminary report, β_3-adrenoceptor null mice (Board *et al*., 2000b). It is also intriguing that higher concentrations of BRL37344, acting via what appeared to be a β_2-adrenoceptor (as the effect was blocked by ICI118551 but not atenolol), inhibited rather than stimulated 2-deoxyglucose uptake in rat soleus muscle (Liu *et al*., 1996a).

β-Adrenoceptors that mediate effects of BRL37344 and other agonists on 2-deoxyglucose uptake in L6 rat skeletal muscle cells have also been characterized. No effects of very low concentrations of BRL37344 were reported, however, and, in contrast to rat skeletal muscle, higher concentrations stimulated 2-deoxyglucose uptake (Tanishita *et al*., 1997; Nevzorova *et al*., 2002, 2006). Nevertheless, as in rat soleus muscle, this effect appeared to be mediated by the β_2-adrenoceptor (Nevzorova *et al*., 2002). Similarly, isoprenaline-stimulated glycogen synthesis in L6 cells displayed a typical concentration–response curve, a typical EC_{50} value of 25 nM and sensitivity to antagonism by ICI118551 (Yamamoto *et al*., 2007).

There are a number of examples of β-adrenoceptors displaying unusual pharmacology (Baker *et al*., 2003a; Arch, 2004; Baker, 2005a, b); it may be that the rat soleus muscle provides another example of this phenomenon. To investigate this pharmacology further, we have studied the effects of not only BRL37344 but also, as the main β-adrenoceptor subtype in soleus muscle is β_2 (Challis *et al*., 1988; Kim *et al*., 1991), the β_2-adrenoceptor agonists clenbuterol and salbutamol. We used soleus muscle from normal mice to facilitate comparison with future studies in genetically modified mice. Similar studies were conducted in a murine muscle cell line in which we could detect β_2-, but not β_1- or β_3-adrenoceptor mRNA by PCR. As fatty acid oxidation and tricarboxylic acid cycle activity may be regulated differently from glucose uptake by β-adrenoceptor agonists and there may be interactions between these metabolic processes, we also studied the effects of the β-adrenoceptor agonists on [1-^{14}C]-palmitate and [2-^{14}C]-pyruvate oxidation in soleus muscle. Our results differ in some respects from results of previous studies in rats (Abe *et al*., 1993; Liu *et al*., 1996a) and wild-type mice of the Aston strain (Board *et al*., 2000a). They broadly show that low concentrations of the agonists do not act via typical β-adrenoceptors. The effects of higher concentrations are blocked by a β_2-adrenoceptor antagonist, but BRL37344 and the selective β_2-adrenoceptor agonists have opposite effects on glucose uptake.

Methods

Animals

Breeding, housing and procedures were conducted in accordance with the UK Government Animals (Scientific Procedures) Act 1986 and approved by The University of Buckingham Ethical Review Board.

Male C57Bl/6 mice (Harlan, Bicester, UK), aged 7–11 weeks, were fed *ad libitum* and killed 3–4 h after the onset of the light cycle, by a UK Government Animals (Scientific Procedures) Act 1986 schedule 1 method. Experiments were conducted over a number of years and during all seasons.

Muscle pre-incubation

The soleus muscle was rapidly dissected from each hind leg and held under resting tension by tying it to a stainless steel clip as described previously (Wang *et al*., 2003). Muscles were immediately placed in Krebs–Henseleit bicarbonate buffer that contained 10 mM HEPES, 5.5 mM glucose and 0.14% (w v^{-1}) fatty acid-free BSA at pH 7.4 and 37 °C. The buffer had been gassed previously with 95% O_2: 5% CO_2 and, after sealing the incubation flasks with Suba rubber stoppers, gassing continued through a needle and vent in the seal while the flasks were shaken (60 r.p.m.) at 37 °C for 60 min.

2-Deoxyglucose-6-phosphate formation

At the end of the pre-incubation, the muscles were transferred to new incubation flasks that contained 3 mL of the same medium, plus 0.1 μCi mL^{-1} 2-deoxy[1-^{14}C] glucose and 0.1 nM bovine insulin, together with various concentrations of β-adrenoceptor agonists and antagonists. (Each muscle was incubated with only one combination of agonist and antagonist concentrations: concentration–response curves were not cumulative.) Following 45 min incubation with continued gassing and shaking, the muscles were rinsed in saline, blotted and frozen in liquid nitrogen.

Incorporation of ^{14}C into 2-deoxyglucose-6-phosphate was determined as described by Liu *et al*. (1996a). Briefly, muscles were digested with 1 M NaOH, neutralized with 1 M HCl and then one portion of the digest was treated with 6% (w v^{-1}) perchloric acid and another with 2.68% (w v^{-1}) $Ba(OH)_2$ and 2.51% (w v^{-1}) $ZnSO_4$. The salts precipitate out 2-deoxyglucose-6-phosphate, so that incorporation of radioactivity into 2-deoxyglucose-6-phosphate can be determined from the difference in radioactivity between the two supernatants.

Palmitate oxidation

At the end of the pre-incubation, the muscles were transferred to flasks that contained 3 mL of the same medium, except that there was no glucose, and the medium

included 0.2 μCi mL^{-1} [1-^{14}C]-palmitate, 0.5 mM palmitate, 0.1 nM insulin and an additional 0.1% fatty acid-free bovine serum, plus β-adrenoceptor agonists and antagonists. The palmitate was prepared as a 5 mM stock solution of palmitic acid with 1% serum albumin. Plastic Pasteur pipettes were partially cut longitudinally from the open end so that the bulbous end could be used to suspend a filter paper soaked with 0.5 mL 1 M NaOH into the flasks when they were sealed. Following 60 min incubation with gassing and shaking, the muscles were removed and weighed, the flasks resealed, and 0.5 and 25% (w v^{-1}) perchloric acid was injected into the medium to drive out CO_2. The flasks were shaken at 70 r.p.m. for 60 min before the filter paper was removed, and radioactivity that had been trapped as $NaH^{14}CO_3$ was counted.

Pyruvate oxidation

At the end of the pre-incubation, the muscles were transferred to flasks that contained 3 mL of the same medium, except that there was no glucose and the medium included 0.5 μCi mL^{-1} [2-^{14}C]-pyruvate, 1 mM pyruvate, 0.1 nM insulin, plus β-adrenoceptor agonists and antagonists. Incubation was for 60 min, and $^{14}CO_2$ production was measured as described for palmitate oxidation.

C2C12 cells

Mouse myogenic clone C2C12 cells (ECACC: 91031101) were obtained from the European Collection of Cell Culture (Sigma Aldrich, Dorset, UK) and maintained in 12-well plates at 2×10^3 cells per cm^2 under 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium supplemented with 15% foetal bovine serum, 2 mM L-glutamine, 10 U mL^{-1} penicillin and 10 μg mL^{-1} streptomycin. Most experiments were conducted with myoblasts. In others, to induce myogenic differentiation, cells that had reached 95–100% confluency were washed in phosphate-buffered saline and cultured in differentiation medium (Dulbecco's modified Eagle's medium supplemented with antibiotics and BSA) for 4 days.

Before measuring 2-deoxyglucose uptake, the cells were first incubated for 2 h with medium (0.5 mL per well) that lacked serum and glutamine. They were then washed three times with Krebs–Henseleit bicarbonate 10 mM HEPES buffer (pH 7.4) and pre-incubated for 1 h with 0.5 mL of this buffer containing 5.5 mM glucose, before being incubated for 15 min at room temperature in the same medium supplemented with 0.2 μCi mL^{-1} 2-deoxy[1-^{14}C] glucose and β-adrenoceptor agonists and antagonists. 2-Deoxyglucose uptake was stopped by adding ice-cold phosphate-buffered saline and washing the cells three times with the same solution. Plates were drained and dried, and the cells solubilized with 0.125 mL 1% Triton X-100 for 20 min prior to counting. Protein content was determined in lysed cells using Bradford reagent.

Expression of β-adrenoceptor subtypes in C2C12 cells

Total RNA was isolated from C2C12 cells, and from C57Bl/6 mouse soleus muscle and interscapular brown adipose tissue as controls for the β-adrenoceptor primers, using Tri-Reagent and isopropanol precipitation. RNA was treated with ribonuclease-free deoxyribonuclease I (Invitrogen Ltd, Paisley, UK). The integrity and loading of the RNA were studied by detection scanning of ribosomal RNA bands (28S and 18S) in agarose gels before and after treatment with deoxyribonuclease I.

First-strand cDNAs were reverse transcribed from 2.5 μg of the total RNA in the presence of oligod(T)$_{12–18}$ primer, 0.5 mM dNTPs, RNase inhibitors (40 U), dithiothreitol (0.01 M) and 200 U of Superscript II RT (Invitrogen) in a total of 20 μL at 42 °C for 45 min. PCRs were performed by mixing 4 μL of first-strand cDNAs, 2.5 mM dNTPs, 0.2 μM gene-specific 5′ and 3′ primers (β_1-adrenoceptor sense: 5′-CAGCATTGAGACCCTGTGTG-3′; β_1-adrenoceptor antisense: 5′-TAGAAGGAGACGACGGACGA-3′; β_2-adrenoceptor sense: 5′-AGCGACTACAAACCGTCACC-3′; β_2-adrenoceptor antisense: 5′-ACCACTCGGGCCTTATTCTT-3′; β_3-adrenoceptor sense: 5′-AGAAACGGCTCTCTGGCTTT-3′; β_3-adrenoceptor antisense: 5′-CTGGTGGCATTACGAGGAGT-3′), and 1 U Taq DNA polymerase (Invitrogen) in a total volume of 20 μL. Twenty-eight cycles of PCR were performed at an annealing temperature of 57.3 °C. PCR products were analysed by agarose gel electrophoresis (1.8% Agarose gel with Gelstar; Cambrex, Wokingham, UK). Bands were quantified using an AlphaImager 1200 system (Flowgen Bioscience Ltd, Nottingham, UK). Quantification of mRNA expression was performed by semiquantitative reverse transcription PCR as described previously (Liu *et al*., 1997), and the level of each mRNA species was normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA.

Data analysis and statistical procedures

Results shown in the same figure panel were obtained in the same experiment using a balanced experimental design. For larger experiments, results were generated over more than 1 day using a single batch of animals, with the treatments divided equally between the days. The six values for BRL37344 in the absence of atenolol shown in Figure 1a are included in the 11 values for BRL37344 in the absence of ICI118551 shown in Figure 1c, because the values for BRL37344 in the presence of ICI118551 were partly obtained in the same experiment as the values for BRL37344 in the presence of atenolol. Other data are from separate experiments. Results are expressed as means ± s.e.mean. Glucose uptake was calculated assuming that 2-deoxyglucose and glucose are not distinguished by the uptake mechanism. Data were analysed by ANOVA followed, if the ANOVA was significant, and Bartlett's test did not show unequal variances ($P<0.05$) by Fisher's least significant difference test. If Bartlett's test showed unequal variances (Figures 4a, 4c and 8a), results were log-transformed before repeating the analysis. In those experiments in which a range of concentrations of β-adrenoceptor agonist was used, comparisons were made only with peak effects of the agonists (for example, 10 pM and 10 nM BRL37344 in Figure 1). Effects of the agonists were analysed by comparing with corresponding baseline values (daggers in the figures) and effects of antagonists by comparing responses at the same concentration of agonist (asterisks in the figures).

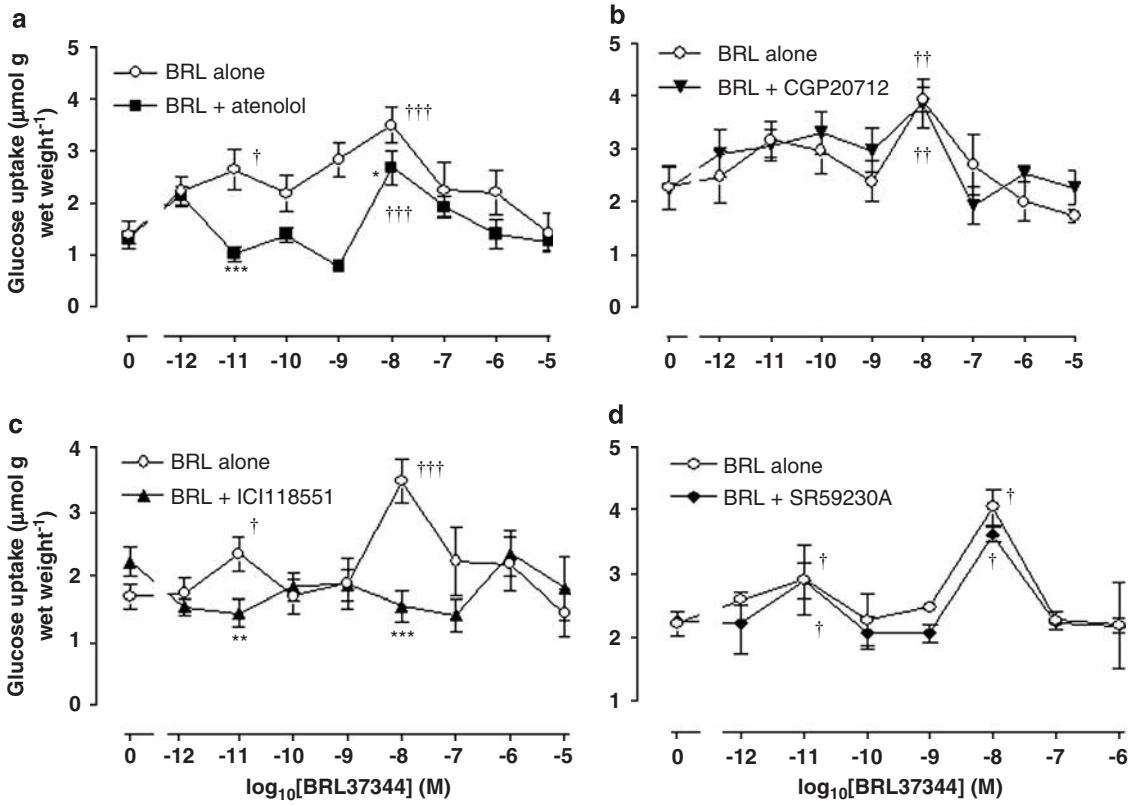

Figure 1 Effect of BRL37344 alone and in the presence of (**a**) 1 μM atenolol ($n=6$), (**b**) 300 nM CGP20712A ($n=6$), (**c**) 1 μM ICI118551 ($n=11$) (**d**) or 1 μM SR59230A ($n=4$ or 5) on glucose uptake in soleus muscle. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to response in the presence of the same concentration of agonist but in the absence of antagonist; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ compared to response in the absence of agonist. $P=0.1$ for the effect of 10 pM compared to zero BRL37344 in panel (**b**).

Drugs and other materials and nomenclature

Radiochemicals were obtained from Amersham International (Amersham, Bucks, UK). BRL37344, sodium salt, clenbuterol, ICI118551 and CGP20712A (1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol dihydrochloride) were obtained from Tocris Cookson Ltd (Bristol, UK). Salbutamol, atenolol, SR59230A, albumin and standard chemicals were obtained from Sigma Aldrich (Gillingham, UK). Nomenclature for receptors and compounds is in accordance with the British Journal of Pharmacology Guide (Alexander *et al*., 2008).

Results

Glucose uptake in soleus muscle in the absence of palmitate

Preliminary experiments (not shown) demonstrated that the rate of 2-deoxy [1-^{14}C]glucose-6-phosphate accumulation (hereafter termed glucose uptake) in soleus muscle was constant up to 60 min of incubation and that the muscle ATP concentration was the same after 0, 45 and 75 min. Glucose uptake was studied in subsequent experiments over 45 min.

As in previous studies using mouse soleus and other rodent skeletal muscles (Abe *et al*., 1993; Liu *et al*., 1996a; Board *et al*., 2000a), there was a peak stimulation of a low concentration of BRL37344 on glucose uptake. The peak effect of BRL37344 in our study was at 10 pM. In contrast to these previous studies, however, a second and larger stimulation of glucose uptake was seen at 10 nM BRL37344 (Figure 1). All but one of these experiments are independent (see Data analysis). We also conducted studies that included 3, 10 and 30 pM or 3, 10 and 30 nM BRL37344, and again peak, statistically significant stimulations of glucose uptake were at 10 pM and 10 nM BRL37344 (data not shown).

Stimulation of glucose uptake by 10 pM BRL37344 was prevented by 1 μM atenolol (selective β_1-adrenoceptor antagonist; Figure 1a). In contrast, 300 nM CGP20712A (highly selective β_1-adrenoceptor antagonist) did not appear to affect glucose uptake in the presence of 10 pM BRL37344, but this was the only experiment in which the effect of BRL37344 did not reach statistical significance (Figure 1b). ICI118551 (1 μM) (selective β_2-adrenoceptor antagonist) prevented the stimulation of glucose uptake by 10 pM BRL37344 (Figure 1c). However, as ICI118551 is highly potent and at 1 μM it may block β_1-adrenoceptors (O'Donnell and Wanstall, 1981; Wilson *et al*., 1984), it was also used at 300 nM, and at this concentration, it did not block the effect of 10 pM BRL37344 (mean glucose uptake in the presence of 300 nM ICI118551 and 10 pM BRL37344 was only 8% lower than that in the presence of BRL37344 alone; data not shown). SR59230A (1 μM) (rodent β_3-adrenoceptor antagonist—see Discussion) (Manara *et al*., 1996) did not affect the response to 10 pM BRL37344 (Figure 1d).

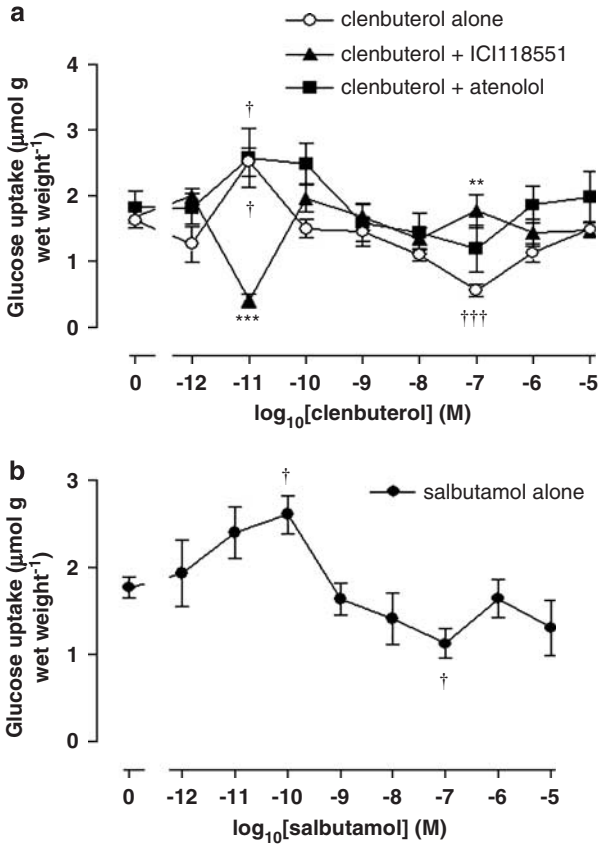

Figure 2 Effects of clenbuterol or salbutamol and of clenbuterol in the presence of 1 μM atenolol or ICI118551 on glucose uptake in soleus muscle. (**a**) Clenbuterol ($n=6$) alone and in the presence of 1 μM atenolol ($n=6$) or 1 μM ICI118551 ($n=6$). (**b**) Salbutamol alone ($n=7$). $^{**}P<0.01$, $^{***}P<0.001$ compared to response in the absence of antagonist; $^{\dagger}P<0.05$, $^{\dagger\dagger\dagger}P<0.001$ compared to response in the absence of agonist.

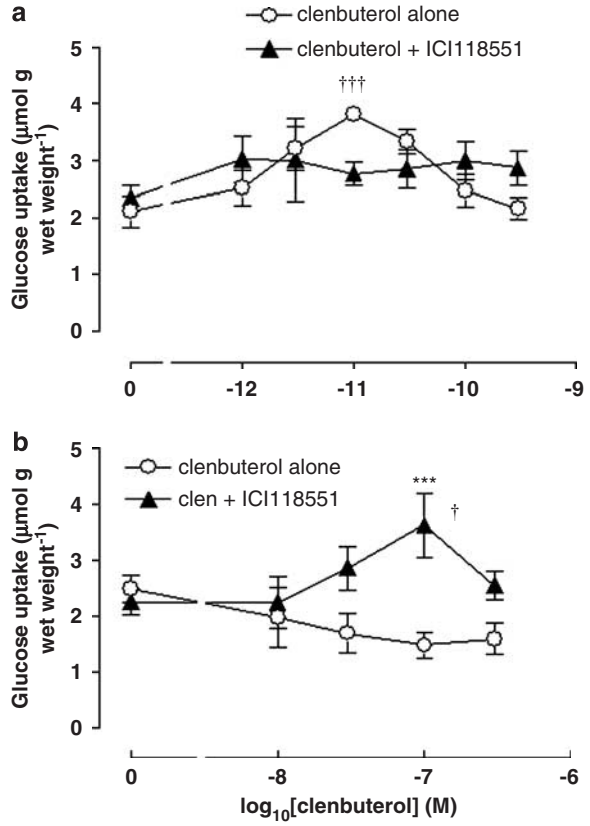

Figure 3 Effects of clenbuterol alone ($n=4$) and in the presence of 300 nM ICI118551 ($n=4$) on glucose uptake in soleus muscle focussing on concentrations around peak effects at (**a**) 10 pM and (**b**) 100 nM. $^{*}P<0.05$, $^{***}P<0.001$ compared to response in the absence of antagonist; $^{\dagger}P<0.05$, $^{\dagger\dagger\dagger}P<0.001$ compared to response in the absence of agonist. $P=0.06$ for effect of 100 nM compared to zero clenbuterol.

The stimulation of glucose uptake by 10 nM BRL37344 was reduced by atenolol but was unaffected by CGP20712A (Figures 1a and b). SR59230A also had no effect (Figure 1d). ICI118551 at concentrations of 1 μM (Figure 1c) and 100 nM (data not shown) prevented any response to 10 nM BRL37344, possibly shifting the effect to higher concentrations of BRL37344.

The β_2-adrenoceptor agonists, clenbuterol and salbutamol, also had biphasic effects on glucose uptake. Like BRL37344, low concentrations stimulated glucose uptake. However, in contrast to BRL37344, high concentrations inhibited glucose uptake (Figure 2).

Stimulation of glucose uptake by the low concentration of clenbuterol (10 pM) was prevented by both 1 μM and 300 nM ICI118551 but not by 1 μM atenolol (Figures 2a and 3a). Similarly, suppression of glucose uptake by the high concentration of clenbuterol (100 nM) was prevented by ICI118551 (Figures 2a and 3b) but not by atenolol (Figure 2a). Indeed, 1 μM ICI118551 transformed the stimulation of glucose uptake by 10 pM clenbuterol into a suppression, and 300 nM ICI118551 transformed the suppression of glucose uptake by 100 nM clenbuterol into a stimulation (Figures 2a and 3b). These results prevented any estimation of the shifts elicited by ICI118551 in the concentration–response curve to clenbuterol.

Palmitate oxidation

BRL37344 (10 nM) stimulated palmitate oxidation. Neither lower nor higher concentrations had any significant effect, though there was a slight trend to stimulation at 10 pM (Figure 4a). In a previous study from this laboratory using wild-type mice of the Aston strain, statistically significant effects of BRL37344 were detected at concentrations of both 100 pM and 10 nM (Board *et al*., 2000a). In a separate experiment, 300 nM ICI118551 suppressed palmitate oxidation in the presence of 10 nM BRL37344 (Figure 4b).

Clenbuterol (10 pM) stimulated palmitate oxidation, but higher concentrations neither stimulated nor suppressed palmitate oxidation (Figure 4a). In a separate experiment, 300 nM ICI118551 suppressed palmitate oxidation in the presence of 10 pM clenbuterol (Figure 4c).

There was a trend for 300 nM ICI118551 to reduce baseline palmitate oxidation in both the BRL37344 and clenbuterol experiments (Figures 4b and c); this was almost statistically significant ($P=0.051$) in the clenbuterol experiment. It may reflect the inverse agonist activity of ICI118551 that has been reported by others (Bond *et al*., 1995; Varma *et al*., 1999; Baker *et al*., 2003b).

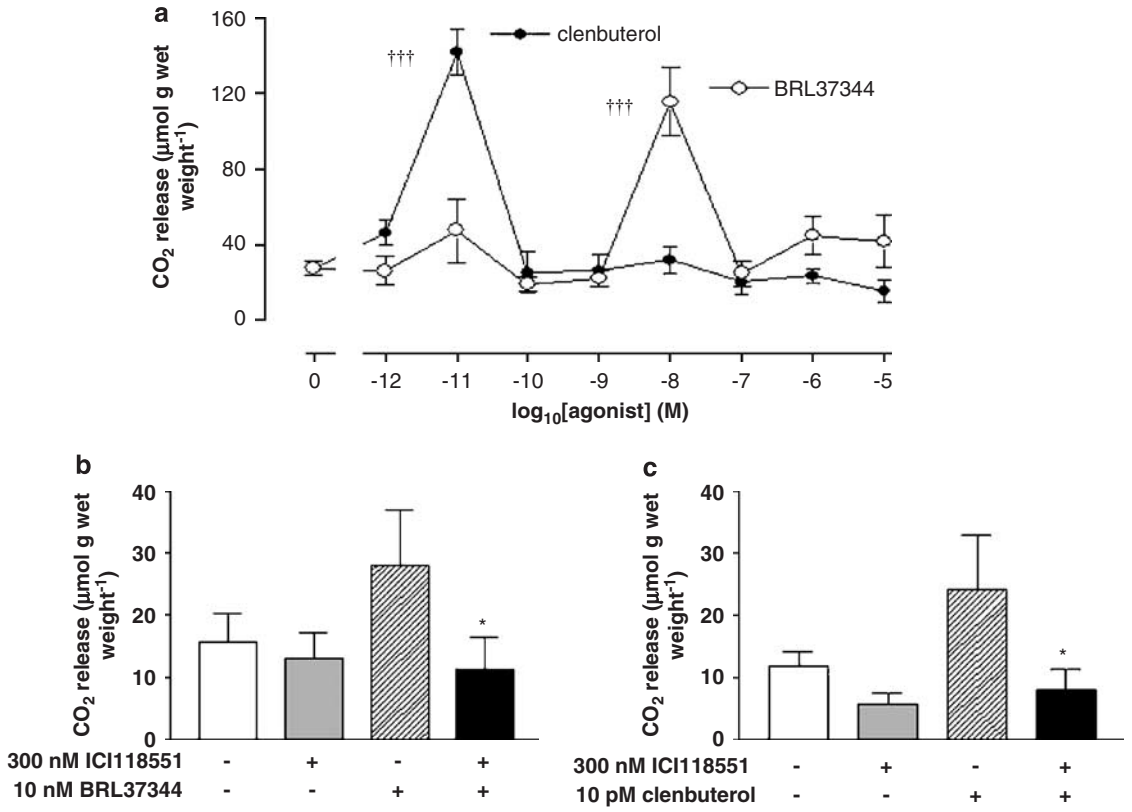

Figure 4 Effects of BRL37344 and clenbuterol on palmitate oxidation in soleus muscle in the absence and presence of 300 nM ICI118551. (**a**) BRL37344 ($n=6$) and clenbuterol ($n=6$) alone. $^{†††}P<0.001$ compared to response in the absence of agonist. (**b**) Effect of 300 nM ICI118551 on response to 10 nM BRL37344 ($n=6$ or 7). (**c**) Effect of 300 nM ICI118551 on response to 10 pM clenbuterol ($n=8$). $^{*}P<0.05$ compared to response in the absence of antagonist ($n=8$). $P=0.051$ for effect of ICI118551 in the absence of clenbuterol in (**c**).

Pyruvate oxidation

The effect of BRL37344 on [2-^{14}C]-pyruvate oxidation, which reflects the rate of the tricarboxylic acid cycle, was studied only at those concentrations that had maximal effects on glucose uptake or palmitate oxidation.

BRL37344 (10 nM) stimulated pyruvate oxidation. In the presence of 300 nM ICI118551, 10 nM BRL37344 did not stimulate pyruvate oxidation significantly ($P=0.058$), though its mean effect was 60% of that in the absence of ICI118551, and ICI118551 did not cause a significant reduction in the response to 10 nM BRL37344 (Figure 5). BRL37344 (10 pM) did not affect pyruvate oxidation (data not shown).

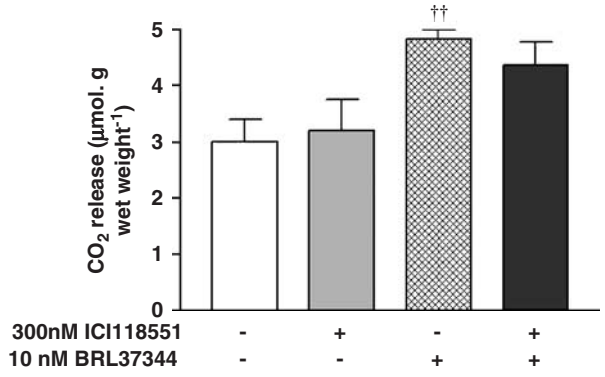

Figure 5 Effect of BRL37344 in the absence or presence of 300 nM ICI118551 on [2-^{14}C]-pyruvate oxidation in soleus muscle ($n=6$ or 7). $^{††}P<0.01$ compared to response in the absence of agonist.

Glucose uptake in the presence of palmitate

Provision of 0.5 mM palmitate as an alternative fuel to glucose appeared to reduce glucose uptake in the absence of the agonists (Figure 6), this effect being statistically significant in the clenbuterol experiment (Figure 6b). The presence of palmitate totally prevented both the low (10 pM) and the high (10 nM) concentrations of BRL37344 from stimulating glucose uptake (Figure 6a). The stimulation of glucose uptake by 10 pM clenbuterol was reduced by 50% and was not statistically significant in the presence of palmitate (Figure 6b). Glucose uptake in the presence of 100 nM clenbuterol was unaffected by the presence of palmitate, but, as palmitate alone suppressed uptake, the suppressive effect of 100 nM clenbuterol was significant only in the absence of palmitate (Figure 6b).

Glucose uptake in C2C12 cells

The effects of BRL37344 and clenbuterol on glucose uptake in C2C12 myoblasts were very similar, but not identical, to those in mouse soleus muscle. BRL37344 (Figure 7a) stimulated glucose uptake at 10 pM and 10 nM, as in soleus muscle.

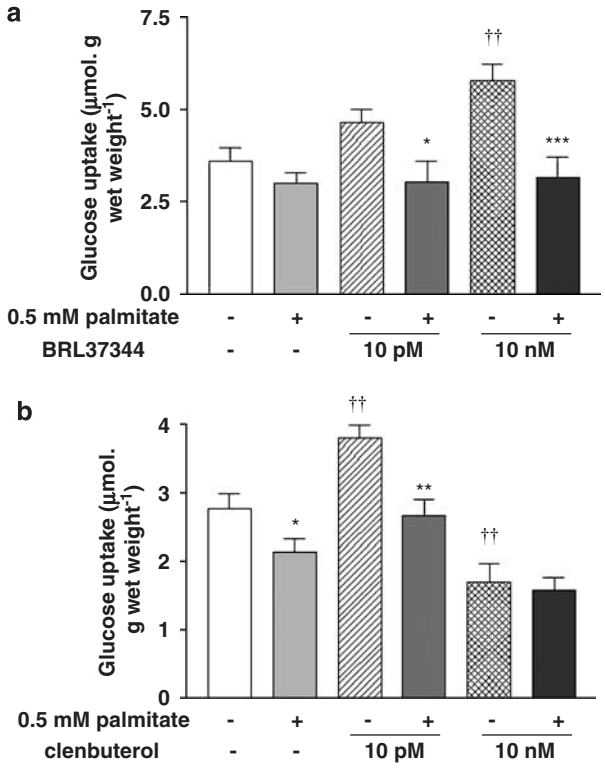

Figure 6 Effect of palmitate on glucose uptake in response to (**a**) BRL37344 ($n=7$ or 8) and (**b**) clenbuterol ($n=5$) in soleus muscle. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to response in the absence of palmitate; $^{\dagger\dagger}P<0.01$ compared to response in absence of agonist.

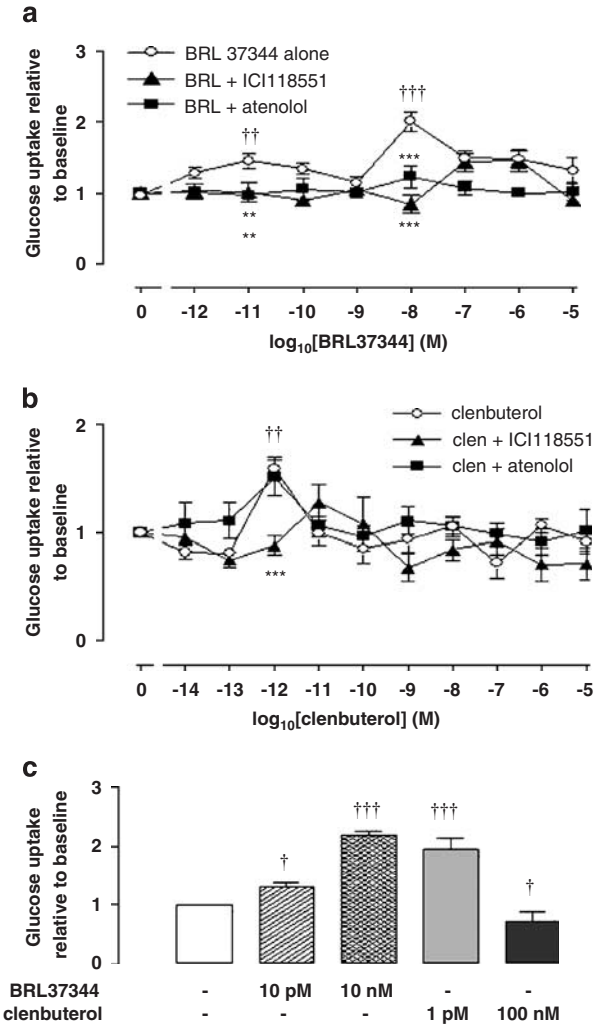

Figure 7 Effects of BRL37344 and clenbuterol on glucose uptake in C2C12 cells. (**a**) BRL37344 and (**b**) clenbuterol alone and in the presence of 1 μM atenolol or ICI118551 in C2C12 myoblasts ($n=5$–7). (**c**) BRL37344 and clenbuterol in C2C12 myotubes ($n=5$ or 6). $^{**}P<0.01$, $^{***}P<0.001$ compared to response in the absence of antagonist. $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ compared to response in the absence of agonist.

Clenbuterol stimulated glucose uptake at 1 pM rather than at 10 pM, but although there was a trend to suppression at 100 nM, this was not statistically significant (Figure 7b). Very similar results were obtained in C2C12 myotubes, except that the suppression of glucose uptake by 100 nM clenbuterol was statistically significant (Figure 7c). As our objective was to model the pharmacology of soleus muscle, further work was conducted with the myoblasts, which provided a more convenient model than myotubes.

The effects of antagonists in C2C12 myoblasts were also similar to those in soleus muscle. Atenolol (1 μM) and 1 μM ICI118551 inhibited the effects of both 10 pM and 10 nM BRL37344 (Figure 7a). ICI118551 (300 nM) was ineffective against 10 pM BRL37344 (Figure 8a). The effect of 1 pM clenbuterol was inhibited by both 1 μM and 300 nM ICI118551 (Figures 7b and 8b), but not by 1 μM atenolol (Figure 7b).

β-adrenoceptor expression in C2C12 cells

Only β_2-adrenoceptor mRNA could be detected by reverse transcription PCR in C2C12 myoblasts and myotubes (Figure 9). All three β-adrenoceptors were detected in brown adipose tissue using the same primers, whereas in soleus muscle, there was mainly β_2-adrenoceptor mRNA and a smaller amount of β_1-adrenoceptor mRNA.

Discussion

Two aspects of the acute effects of BRL37344 and clenbuterol on metabolism in murine soleus muscle and C2C12 cells will be considered: the nature of the receptors that mediate these effects and the interactions between the metabolic processes that are altered.

Receptors in muscle that mediate responses to low concentrations of agonists

Our results agree with previous studies on mice (Aston and C57Bl/6 strains) and rats (Wistar and Sprague–Dawley strains) in the finding that a concentration of BRL37344 (10 pM) that is 100-fold below its EC_{50} value for stimulation of lipolysis in rat adipocytes via the β_3-adrenoceptor (Arch *et al*., 1984) stimulates glucose uptake in soleus muscle by

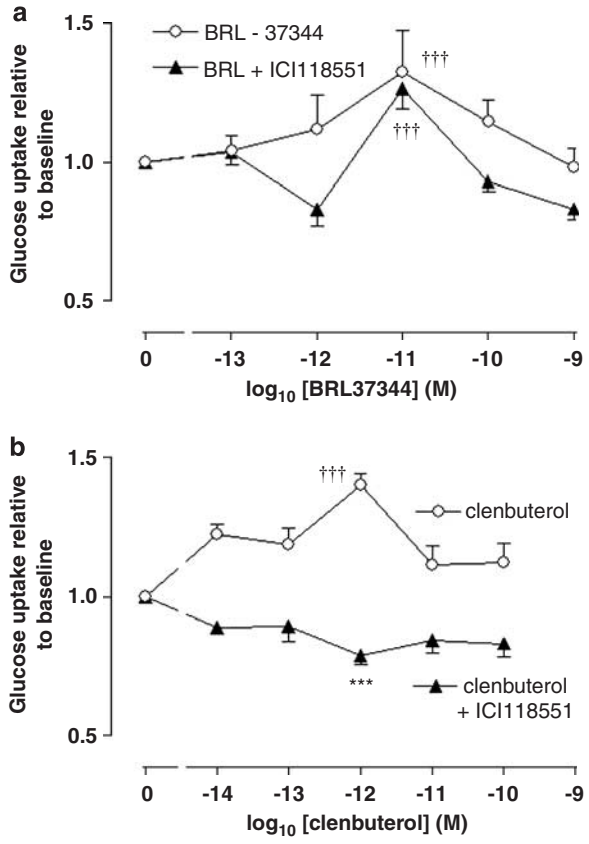

Figure 8 Effects of (**a**) BRL37344 or (**b**) clenbuterol alone and in the presence of 300 nM ICI118551 on glucose uptake in C2C12 cells ($n=6$–8). ***$P<0.001$ compared to response in the absence of antagonist; $^{\dagger\dagger\dagger}P<0.001$ compared to response in the absence of agonist.

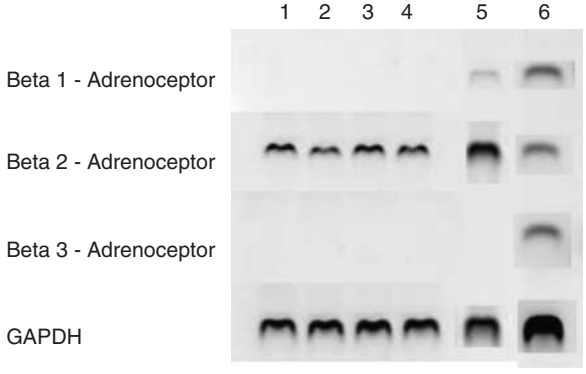

Figure 9 β-Adrenoceptor gene expression in C2C12 cells. Total RNA was isolated from C2C12 myotubes (lanes 1 and 3) and myoblasts (lanes 2 and 4), and (as controls for the primers) soleus muscle (lane 5) and brown adipose tissue (lane 6) from C57Bl/6 mice. The RNA was subjected to reverse transcription followed by PCR with mouse β-adrenoceptor- and GAPDH-specific primers. The pictures are representative of $n=6$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

about 30–60% (Abe *et al*., 1993; Liu *et al*., 1996a; Board and Cawthorne, 1998; Board *et al*., 2000a).

As in rat soleus muscle (Liu *et al*., 1996b), this effect of 10 pM BRL37344 was not blocked by the rodent β_3-adrenoceptor antagonist SR59230A (1 μM), consistent with a report that very low concentrations of BRL37344 stimulated pyruvate and palmitate oxidation in soleus muscle of β_3-adrenoceptor null mice (Board *et al*., 2000b). SR59230A may not be a selective antagonist of human β_3-adrenoceptors, in particular human cloned β_3-adrenoceptors (Arch, 2000), and it is a partial agonist of the human and mouse cloned β_3-adrenoceptors (Baker, 2005a) and of the β_3-adrenoceptor in some rodent tissues (Dumas *et al*., 1998; Horinouchi and Koike, 2001; Brahmadevara *et al*., 2003). In 3T3-F442A adipocytes, it was an antagonist of disodium (*R*,*R*)-5-(2-[{2-(3-chlorophenyl)-2-hydroxyethyl}-amino]propyl)-1,3-benzodioxole-2,2,dicarboxylate (CL316243)-stimulated cAMP accumulation, but an agonist of the extracellular acidification rate (Hutchinson *et al*., 2005). Nevertheless, 1 μM SR59230A, for which reported pK_B values are mostly close to or greater than 8.0, clearly blocks β_3-adrenoceptors (Manara *et al*., 1996; Nisoli *et al*., 1996; Hutchinson *et al*., 2001, 2005) in many rodent tissues, and it did not behave as a partial agonist that stimulated glucose uptake in the present study.

The effect of 10 pM BRL37344 was blocked by 1 μM atenolol, for which pA_2 values of 7.1, 7.6 and 7.6 have been reported for antagonism of β_1-adrenoceptors in mouse, rat and guinea pig tissues (Henry and Goldie, 1990; Arch and Kaumann, 1993). This raised the possibility that 10 pM BRL37344 acted via the β_1-adrenoceptor. However, other workers have reported no effect of 1 μM atenolol (Liu *et al*., 1996a; Board *et al*., 2000a). Moreover, pK_B/pA_2 values similar to those of atenolol (7.4 and 7.3) have been reported for antagonism of β_1-adrenoceptors by SR59230A in rat colon and mouse ileum (Manara *et al*., 1996; Hutchinson *et al*., 2001), and SR59230A had no effect. CGP20712A (300 nM), a 100-fold more potent rodent β_1-adrenoceptor antagonist (Kaumann, 1986; Zwaveling *et al*., 1996), also appeared to have no effect, but in this experiment, the effect of 10 pM BRL37344 did not reach statistical significance.

Atenolol (1 μM) (Chin and Pennefather, 1992; Arch and Kaumann, 1993; Suh *et al*., 1999; Pourageaud *et al*., 2005), 1 μM SR59230A (Nisoli *et al*., 1996; Yurtcu *et al*., 2006) and 300 nM CGP20712A would not be expected to block rodent β_2-adrenoceptors. ICI118551 is, in contrast, a highly potent and selective β_2-adrenoceptor antagonist with reported pA_2 values in rat tissues of greater than 9 (Bilski *et al*., 1983; Vidal-Beretervide and Castaneda, 1988; Pourageaud *et al*., 2005). A pA_2 value of 8.7 has been reported for antagonism of adrenaline in a mouse pineal tumour cell line (Suh *et al*., 1999). We initially used 1 μM ICI118551 because in other studies this concentration did not block the effect of the low BRL37344 concentration (Liu *et al*., 1996a; Board *et al*., 2000a), but we found that it did. However, 300 nM ICI118551, which should be more than sufficient to block the β_2-adrenoceptor, did not antagonize the effect of 10 pM BRL37344.

Thus, the pharmacology of the receptor that mediated the response to 10 pM BRL37344 was not that of any typical β-adrenoceptor. Considering the blockade by atenolol, 16–21% of β-adrenoceptors in rat soleus muscle are β_1-adrenoceptors (Kim *et al*., 1991), and β_1-adrenoceptors are known to be capable of displaying an atypical pharmacology previously

ascribed to a 'β_4-adrenoceptor' (Konkar *et al*., 2000; Kaumann *et al*., 2001) and now to a low-affinity site of the β_1-adrenoceptor, but this pharmacology is very different. For example, the 'β_4-' but not the skeletal muscle adrenoceptor is stimulated by CGP-12177 (Board *et al*., 2000a), and the 'β_4-adrenoceptor' is not stimulated by BRL37344 (Kaumann and Molenaar, 1996, 2008). Moreover, the 'CGP-12177 site' of the human β_1-adrenoceptor (equivalent to the 'β_4-adrenoceptor') is not blocked by atenolol (Baker *et al*., 2003a). BRL37344 is also a muscarinic and α_1-adrenoceptor antagonist, but with pEC_{50} values of <6 and <5 respectively (Vrydag and Michel, 2007). Moreover, our unpublished data show that acetylcholine stimulates glucose uptake in soleus muscle, consistent with work by others in C2C12 cells (Liu *et al*., 2002). We also cannot exclude the possibility that the effect of 10 pM BRL37344 is mediated by an intracellular receptor.

Low concentrations of the selective β_2-adrenoceptor agonists clenbuterol and salbutamol, like 10 pM BRL37344, also stimulated glucose uptake. The peak effects of these compounds were at concentrations 250-fold lower than their EC_{50} values for relaxation of rat uterus or guinea pig trachea (see Table III of Arch and Kaumann, 1993). In contrast to the response to 10 pM BRL37344, stimulation of glucose uptake by 10 pM clenbuterol was prevented by 300 nM ICI118551 and was not prevented by 1 μM atenolol. The stimulation of palmitate oxidation by 10 pM clenbuterol was also prevented by 300 nM ICI118551. The receptor is therefore like the β_2-adrenoceptor in terms of its response to antagonists, but it is responsive to exceptionally low concentrations of β_2-adrenoceptor agonists. β_2-Adrenoceptors that display atypical pharmacology have been described before, but they do not display this extreme sensitivity to agonists (Heubach *et al*., 2003; Baker *et al*., 2003c; Teixeira *et al*., 2004).

β-Adrenoceptor agonists elicited a small increase in lipolysis in mice that lack β_1-, β_2- and β_3-adrenoceptors (Tavernier *et al*., 2005), but the mediator of this response had low affinity for the agonists, in contrast with the high-affinity site in skeletal muscle described here. Despite their different responses to atenolol and ICI118551, it is possible that the same site mediates responses to very low concentrations of BRL37344 and clenbuterol if these agonists bind to different sites or conformations of a receptor (Rimele *et al*., 1988; Konkar *et al*., 2000; Baker *et al*., 2003a, c; Baker, 2005a; Hutchinson *et al*., 2005; Sato *et al*., 2007).

Receptors in muscle that mediate responses to high concentrations of agonists

BRL37344 at the higher concentration of 10 nM also stimulated glucose uptake, roughly doubling the rate. Atenolol, CGP20712A and SR59230A did not inhibit this stimulation, whereas ICI118551 did. It seemed to shift the stimulations to higher concentrations of BRL37344. The shifts in the peak response to BRL37344 elicited by 1 μM and 100 nM ICI118551 were very broadly consistent with a pA_2 value for ICI118551 of 8.7 when antagonizing adrenaline in a mouse pineal tumour cell line (Suh *et al*., 1999). These findings and reported EC_{50} values of about 40 nM for BRL37344 in rat and guinea pig tissues (Arch and Kaumann, 1993) suggest the involvement of a β_2-adrenoceptor. This conclusion must be reconciled, however, with the finding that 100 nM clenbuterol, a selective β_2-adrenoceptor agonist, inhibited, rather than stimulated, glucose uptake and this effect was similarly prevented by ICI118551 but not atenolol. Salbutamol (100 nM), another β_2-adrenoceptor agonist, also inhibited glucose uptake. Either the β_2-adrenoceptor did not mediate the response to both agonists or it is able to mediate opposing effects, depending on the agonist.

The latter explanation could be an example of a phenomenon known as 'agonist-receptor trafficking' (Kenakin, 1995) or 'ligand/agonist-directed signalling', which has been described previously for β_2- and β_3-adrenoceptors (Baker *et al*., 2003b; Sato *et al*., 2007). It could be due to clenbuterol and salbutamol being more able than BRL37344 to promote coupling of the β_2-adrenoceptor to G_i or a G protein-independent mechanism (Hasseldine *et al*., 2003; Baker *et al*., 2003b; Heubach *et al*., 2004). The possibility that clenbuterol and salbutamol have higher efficacy than BRL37344 with respect to β_2-adrenoceptor-mediated Gi signalling is especially interesting. Ligands can stimulate or reduce cAMP production in cells that express cloned β_2-adrenoceptors depending on the conditions (Chidiac *et al*., 1994), and our unpublished data show that 100 nM clenbuterol reduces soleus muscle cAMP content, whereas 100 nM BRL37344 does not affect cAMP, consistent with published data for rat soleus muscle (Roberts and Summers, 1998). It may be significant that in the presence of 300 nM ICI118551, 100 nM clenbuterol behaved like 10 nM BRL-37444 and stimulated glucose uptake (Figure 4b). In other words, 300 nM ICI118551 appeared to reveal a stimulatory effect of clenbuterol. Thus, a possible explanation is that clenbuterol, but not BRL37344, directs β_2-adrenoceptor signalling to Gi and that ICI118551 prevents this linkage (Baker *et al*., 2003b, 2003c), while allowing clenbuterol to use whatever signalling mechanism BRL37344 uses to stimulate glucose uptake. Another possibility is that a signalling mechanism is constitutively active and is reduced by clenbuterol or BRL37344. In other words, this compound is an inverse agonist, the other being an agonist. Our unpublished data referred to above do not, however, show opposing effects of the compounds on the concentration of cAMP.

Board and Cawthorne (1998) described a stimulatory effect of 10 nM BRL37344 on glucose uptake in soleus muscle from genetically obese (Lep^{ob}/Lep^{ob}) C57Bl/6 mice, and Nevzorova *et al*. (2002) described a stimulatory effect in rat L6 skeletal muscle cells. Both effects were antagonized by ICI118551 (1 and 0.1 μM in muscle and L6 cells respectively). Stimulation of cAMP accumulation by isoprenaline in rat soleus muscle was also sensitive to ICI118551 (Roberts and Summers, 1998). Other reports, however, describe inhibitory effects of 10 nM or higher concentrations of BRL37344 on glucose uptake in soleus muscle that are also antagonized by 1 μM ICI118551 (Liu *et al*., 1996a; Board *et al*., 2000a). In each case, it was argued that the β_2-adrenoceptor mediated these effects.

Nanomolar but not picomolar concentrations of BRL37344 and other β_3-adrenoceptor agonists stimulate glucose uptake in brown and white adipocytes through a

cAMP-dependent mechanism (Ohsaka *et al*., 1998; Chernogubova *et al*., 2004, 2005). Roberts and Summers (1998) raised the possibility that previously reported effects of BRL37344 on glucose uptake in skeletal muscle were due to the presence of infiltrating adipocytes. This interpretation is consistent with the discovery of mRNAs for both white and brown adipose tissue proteins in skeletal muscle, as well as identification of adipocytes by morphometric analysis of skeletal muscle (Evans *et al*., 1996; Nagase *et al*., 1996; Almind *et al*., 2007). As Roberts and Summers (1998) pointed out, however, if rat soleus muscle contains a sufficient number of adipocytes to account for the effect of BRL37344 on glucose uptake in previous studies, one might expect it to increase cAMP accumulation, but it did not do this in their experiments. Moreover, stimulation of glucose uptake by BRL37344 in brown and white adipocytes is mediated by the β_3-adrenoceptor (Chernogubova *et al*., 2005), but our studies and those of others (Board and Cawthorne, 1998; Nevzorova *et al*., 2002) show that concentrations of BRL37344 of the order of 10 nM do not stimulate glucose uptake in soleus muscle and L6 cells via the β_3-adrenoceptor. It is therefore unlikely that stimulation of glucose uptake by BRL37344 in soleus muscle was due to the presence of adipocytes in muscle.

C2C12 cells

The effects of the agonists and antagonists on glucose uptake in C2C12 cells were surprisingly similar to those in soleus muscle. We could detect β_2-, but not β_1- or β_3-adrenoceptor mRNA in C2C12 myoblasts and myotubes, suggesting either that all the responses to the β-adrenoceptor agonists were mediated by β_2-adrenoceptors or that some—most likely those to pM concentrations of BRL37344—were mediated by non-β-adrenoceptors.

In contrast to our studies in C2C12 cells, studies in rat L6 myocytes have revealed normal concentration–glucose uptake response curves to BRL37344, isoprenaline and the $\beta_{2/3}$-adrenoceptor agonist zinterol (Hutchinson *et al*., 2006). Zinterol only stimulated uptake, unlike clenbuterol and salbutamol, in our study. As the concentration–response curves were of a classical shape in L6 myocytes, it was possible to demonstrate that K_B values for antagonists, including ICI118551, were consistent with each agonist acting via the β_2-adrenoceptor. There was no evidence of stimulation of glucose uptake by very low concentrations of agonists (Tanishita *et al*., 1997; Nevzorova *et al*., 2002). Similarly, there was no evidence that very low concentrations of isoprenaline stimulated glycogen synthesis in L6 cells (Yamamoto *et al*., 2007). Thus C2C12 cells, even as myoblasts, are a better model than L6 cells of soleus muscle responses to BRL37344.

Shape of concentration–response curves

The 'sharpness' of the concentration–response curves to the agonist is surprising: for example, most of the concentration–response curves show no effect of BRL37344 at concentrations 10-fold above or below those that elicited peak effects. As 10 nM BRL37344 stimulated glucose uptake, we cannot easily argue as others have done (Liu *et al*., 1996a; Board *et al*., 2000a) that 100 pM and 1 nM BRL37344 had no effect because a stimulatory response mediated by an atypical receptor was opposed by an inhibitory effect mediated by a classical β_2-adrenoceptor. It seems more likely that ligand-directed signalling is sensitive to ligand concentration.

Metabolic interactions

We studied [1-^{14}C]-palmitate oxidation and [2-^{14}C]-pyruvate oxidation (a measure of tricarboxylic acid cycle activity) because fatty acid oxidation inhibits glucose utilization, and citrate, produced by the tricarboxylic acid cycle, plays a role in orchestrating this link by inhibiting the activity of fructose-1:6-bisphosphatase (Randle, 1998; Lind, 2004). A U-shaped concentration–response curve for the effect of the peroxisome proliferator-activated receptor-β agonist 2-methyl-4([(4-methyl-2-[4-trifluoromethylphenyl]-1,3-thiazol-5-yl)methyl)sulphanyl]phenoxy)acetic acid (GW501516) on glucose oxidation in rat soleus muscle has been attributed to switching of fuel utilization from lipid to carbohydrate as the concentration of the agonist increases (Brunmair *et al*., 2006).

Although it did not have a statistically significant effect at 10 pM, the concentration–response curve for the effect of BRL37344 on palmitate oxidation in soleus muscle (Figure 5) was broadly similar to that for glucose uptake (Figure 1), and the effects of 10 nM BRL37344 on both glucose uptake and palmitate oxidation were inhibited by 300 nM ICI118551, suggesting the involvement of a common receptor mechanism. BRL37344 (10 nM) also stimulated [2-^{14}C]-pyruvate oxidation, indicating that tricarboxylic acid cycle activity was increased.

The presence of palmitate prevented the stimulation of glucose uptake by both 10 pM and 10 nM BRL37344. Thus BRL37344 stimulated fat oxidation in preference to carbohydrate metabolism. The presence of palmitate did not cause 10 nM BRL37344 to suppress glucose uptake, however, suggesting that the suppression of glucose uptake by 100 nM clenbuterol cannot be simply a consequence of it stimulating fat oxidation.

This conclusion is supported by the following findings: 100 nM clenbuterol neither stimulated nor inhibited palmitate oxidation; palmitate did not enhance the suppression of glucose uptake by 100 nM clenbuterol; 100 nM clenbuterol suppressed glucose uptake in soleus muscles of mice that had been fasted overnight to reduce lipid stores (results not shown). Thus, 100 nM clenbuterol (and salbutamol) appear to couple the β_2-adrenoceptor to mechanisms that suppress glucose uptake but have no effect on palmitate oxidation. In contrast, 10 nM BRL37344 couples a β-adrenoceptor to mechanisms that stimulate both glucose uptake and palmitate oxidation.

In conclusion, this work has extended our understanding of the peculiar pharmacology of the metabolic effects of β-adrenoceptor agonists in soleus muscle. Nanomolar concentrations of BRL37344 and clenbuterol affect glucose uptake via receptors that are antagonized by a β_2-adrenoceptor antagonist. However, clenbuterol reduces glucose

uptake without affecting palmitate oxidation, whereas BRL37344 stimulates both processes. Picomolar concentrations of the agonists affect metabolism via an undefined receptor or receptors. The molecular nature of these receptors and their intracellular signalling mechanisms will be the subject of future reports.

Acknowledgements

RAN was supported by a bursary from the Ghanaian government. We thank Monique Heald for critical reading of the paper.

Conflict of interest

J Arch and M Cawthorne consult various pharmaceutical companies but have no dealings with these companies that present any conflict with the results in this paper.

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