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BRL37344, but not CGP12177, stimulates fuel oxidation by soleus muscle in vitro

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Abstract

The β_3-adrenoceptor agonist, (*RR* + *SS*)-(±)-4-[2-)2-)3-chlorophenyl)-2-hydroxyethyl)amino)propyl]phenoxyacetate (BRL37344), stimulated fuel utilisation by isolated mouse soleus muscle at concentrations 10- to 100-fold lower than those required to stimulate lipolysis in brown adipocytes. At 1×10^{-10} M BRL37344, uptake and phosphorylation of 2-deoxyglucose was increased (40%), as was glucose-oxidation (50%), palmitate-oxidation (70%) and oxidation of [2-^{14}C]pyruvate (2-fold), indicating stimulation of tricarboxylic acid cycle reactions. Oxidation of [1-^{14}C]pyruvate was unaffected, indicating no stimulation of pyruvate dehydrogenase activity. Other β_3-adrenoceptor agonists, disodium(*RR*)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino]propyl]-1,3-benzodioxazole-2,2-dicarboxylate (CL316,243, 1×10^{-7} M) and (*S*)-4-{2-[2-hydroxy-3-(4-hydroxyphenoxy)propylamino]ethyl}phenoxymethylcyclohexylphosphiric acid lithium salt (SB226552, 1×10^{-9} M), achieved similar stimulation of 2-deoxyglucose uptake and phosphorylation but (±)-4-(3-*t*-butylamino-2-hydroxypropoxy)benzimidazol-2-one (CGP12177A) had no effect. The inhibitor of protein kinase A, H-89 (isoquinolinesulfonamide), had little effect on the stimulation of pyruvate-oxidation by BRL37344, while the specific inhibitor of protein kinase C, bisindolylmaleimide IX, reduced the stimulated rate to slightly below basal values. We consider that these responses provide evidence of the presence of a novel β-adrenoceptor in skeletal muscle, which we have termed β_{skel}-adrenoceptor.

Keywords: β_3-Adrenoceptor agonists; Thermogenesis; Pyruvate-oxidation; Glucose metabolism; Soleus muscle, isolated

1. Introduction

Adrenoceptors have received a great deal of attention because of their strong influence over fuel consumption and energy expenditure by a wide range of tissues. Such properties are of particular relevance to a study of the development of obesity although the relationship between adrenoceptor action and energy expenditure in whole body terms is a complex one. The β_1- and β_2-adrenoceptors have long established roles concerning circulation and fuel mobilisation, but since the discovery of the rodent adipocyte β_3-adrenoceptor (Arch et al., 1984) and the characterisation of its importance in thermogenesis, the potential significance of such receptors has widened.

The present work focuses on a little-characterised β-adrenoceptor present in skeletal muscle, which for the purposes of the current study, we have termed β_{skel}-adrenoceptor. Since skeletal muscle represents a large proportion of the body's respiring mass, it is considered that any factor influencing the oxidative metabolism of this tissue could have a significant impact on whole body energy homeostasis.

The existence of β_{skel}-adrenoceptor has been inferred from previous work reporting effects of (*RR* + *SS*)-(±)-4-[2-)2-)3-chlorophenyl)-2-hydroxyethyl)amino)propyl]phenoxyacetate (BRL37344) on glucose uptake by rat skeletal muscle (Abe et al., 1993; Liu et al., 1996). Such effects were achieved at concentrations 10- to 100-fold lower than those required to elicit lipolysis by brown adipocytes. The present study extends these earlier studies and uses whole

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isolated soleus muscle from adult mice rather than soleus muscle of very young rats or a stripped muscle preparation. Concentrations of the β_3-adrenoceptor agonist, BRL37344, required to stimulate β_{skel}-adrenoceptor in such a system have been identified and effects on the uptake and phosphorylation of 2-deoxyglucose and on the oxidation of glucose, palmitate and pyruvate (labelled at the C1 and C2 positions) under similar conditions have been assessed. The effects of several other agonists of the β_3-adrenoceptor, disodium(*RR*)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino]propyl]-1,3-benzodioxazole-2,2-dicarboxylate (CL316,243), (*S*)-4-{2-[2-hydroxy-3-(4-hydroxyphenoxy)propylamino]ethyl}phenoxymethylcyclohexylphosphiric acid lithium salt (SB226552) and (±)-4-(3-*t*-butylamino-2-hydroxypropoxy)benzimidazol-2-one (CGP12177A), on 2-deoxyglucose uptake and phosphorylation have also been measured for comparative purposes. In addition, a preliminary attempt has been made to indicate possible intracellular linkage of β_{skel}-adrenoceptor using inhibitors of protein kinase A and of protein kinase C.

2. Materials and methods

2.1. Materials

2.1.1. Animals

Wild-type (+/+) mice of the Aston strain of *ob/ob* mice were maintained as a breeding colony at the Clore Laboratory, Buckingham, UK. Animals were fed ad libitum and maintained on a 12-h dark/12-h light cycle and used when aged 12–14 weeks. Preliminary studies showed that there was no age-related change in the response to BRL37344 on 2-deoxyglucose uptake and phosphorylation. The mice were killed by cervical dislocation 2 h after the onset of the light cycle. Animal breeding, housing and procedures were carried out in accordance with the UK Government Animals (Scientific Procedures) Act 1986.

2.1.2. Consumables

All radiolabelled chemicals, except [2-^{14}C]pyruvate, were purchased from Amersham International, Amersham, UK and [2-^{14}C]pyruvate from NEN, Zaventem, Belgium. BRL37344 and ICI118551 (D-(±)-1-(7-methylinden-4-yloxy)-3-isopropylaminobutan-2-ol) were obtained from Tocris Cookson, Bristol, UK; CGP12177A from Research Biochemicals International, Natick, MA, USA; H-89 (isoquinolinesulfonamide) from Calbiochem, Nottingham, UK and bisindolylmaleimide IX from Alexis Biochemicals, Nottingham, UK. CL316,243 and SB226552 were generous gifts from the American Home Products and SmithKline Beecham Pharmaceuticals, respectively. All other reagents came from Merck, Lutterworth, UK or from Sigma, Poole, UK.

2.2. Methods

2.2.1. Incubation of isolated soleus muscle

Proximal and distal tendons of the soleus muscle were ligated, the muscle removed from the mouse hind leg and secured, by means of the ligatures, to a stainless steel clip under a tension designed to approximate that of the resting muscle (Espinal et al., 1983). The muscle and clip were immediately placed in a silicone-treated flask containing a 3-ml volume of Krebs Henseleit Bicarbonate (KHB) medium (previously gassed for 15 min with 95% O_2:5% CO_2), pH 7.4, containing 0.14% essentially fatty acid free bovine serum albumin and 5.5 mM glucose and pre-incubated for 30 min at 37°C. Throughout the pre-incubation and incubation periods, a throughflow of 95% O_2:5% CO_2 was maintained, with constant shaking (70 strokes/min), in order to achieve fully oxygenated conditions.

At the end of the pre-incubation period, muscle plus clip were quickly rinsed in KHB at 37°C and transferred to a new flask containing a 3-ml volume of an appropriate incubation medium consisting of KHB–bovine serum albumin and substrate with additions of agonists and antagonists as indicated in the results.

Measurements of substrate oxidation were made in the presence of 5.5 mM glucose (+15 Bq/ml D-[U-^{14}C]glucose) or 1 mM pyruvate (+2.3 Bq/ml [1-^{14}C] or [2-^{14}C]pyruvate) or palmitate–bovine serum albumin complex, giving a final concentration of 1 mM palmitate (+6.2 Bq/ml [1-^{14}C]palmitate). All incubations contained insulin at 1 μU/ml, which is a sub-stimulatory concentration and is present in order to maintain tissue viability.

2.2.2. Measurements of 2-deoxyglucose uptake and phosphorylation

For measurements of rates of uptake and phosphorylation of 2-deoxyglucose, the KHB–bovine serum albumin medium was supplemented with 5.5 mM 2-deoxyglucose (Dimitriadis et al., 1997) containing 15 Bq/ml^{-1} [1-^{14}C]2-deoxyglucose.

At the end of the 45-min incubation period, the muscle was removed from the clip and quickly frozen in liquid N_2. Alkali digestion of each muscle was achieved by heating at 60°C for 45 min in a 0.5-ml volume of 1 M NaOH. Digested samples were neutralised with 0.5 M HCl and 0.3 ml aliquots added to 1 ml of 6% $HClO_4$ (to measure total radioactivity: ([^{14}C]2-deoxyglucose + [^{14}C]2-deoxyglucose-6-phosphate)) and to 1 ml of a 1:1 mixture of saturated $Ba(OH)_2$ and 2.5% $ZnSO_4$ (to measure radioactivity contained in the [^{14}C]2-deoxyglucose fraction only) (Issad et al., 1987). Samples were centrifuged (10 min at 3000 × *g*) and the radioactivity in the supernatants measured. Subtraction of the $BaSO_4$ fraction from that of the total gives the amount of radioactivity present as [^{14}C]2-deoxyglucose-6-phosphate only.

This method enables the rate of flux of 2-deoxyglucose to 2-deoxyglucose-6-phosphate to be measured, encom-

passing reactions catalysed by the glucose transporter (predominantly GLUT1 at low insulin concentration) and hexokinase. Preliminary tests showed that rates of flux remained linear during the 45 min of incubation in the isolated muscle system used here. Thus, build-up of 2-deoxyglucose-6-phosphate to levels where hexokinase might be inhibited was avoided. Results of incubations in the presence of 5.5 mM glucose plus a trace of [^{14}C]2-deoxyglucose achieved similar rates of uptake and phosphorylation to those containing 2-deoxyglucose only (results not shown).

2.2.3. Preparation of palmitate–bovine serum albumin complex

For the purposes of measuring rates of oxidation of palmitate, a complex was prepared using bovine serum albumin. To a solution of 0.9% NaCl plus 10 mM HEPES, bovine serum albumin was added to give a final concentration of 10%. Sufficient palmitate (from a solution in ethanol) was added to the bovine serum albumin suspension to give a final concentration in the stock solution of 23 mM, together with [1-^{14}C]palmitate to 0.15 Bq/ml. The resulting suspension was incubated at 37°C overnight in a shaking water bath. The palmitate–bovine serum albumin complex was stored at −20°C and aliquots were incubated at 37°C for at least 4 h prior to use.

2.2.4. Collection of $^{14}CO_2$ for measurements of substrate oxidation

After 90 min of incubation, 0.6 ml of 25% $HClO_4$ was injected into the incubation medium through the flask stopper. At the same time, 0.2 ml of a 1:1 mixture of methanol and phenylmethylamine was injected into wells incorporated in the stoppers. Flasks were shaken for a further 60 min at room temperature after which the wells were carefully removed and their radioactivity measured (Curi et al., 1988).

2.2.5. Statistics

Each incubation with a particular drug concentration was replicated at least five times and results are presented as mean values ± S.E.M. The statistical significance of differences between control and experimental groups was assessed using analysis of variance (ANOVA). Multiple

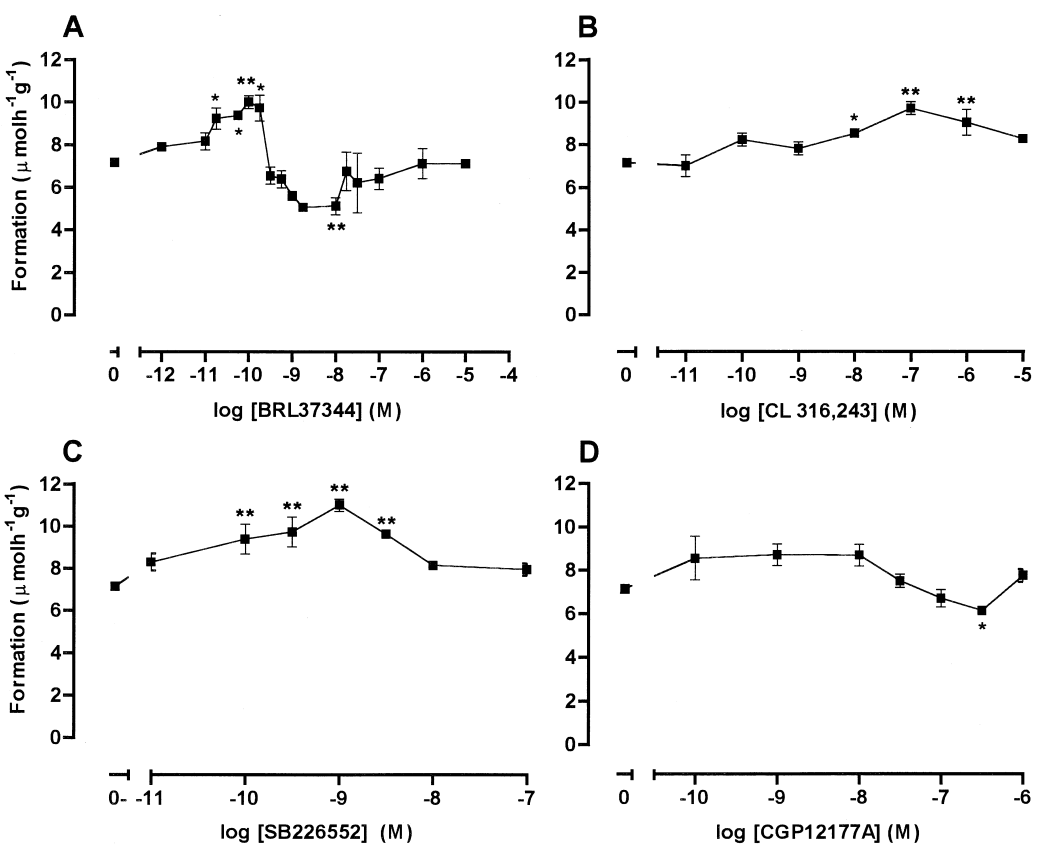

Fig. 1. Formation of 2-deoxyglucose-6-phosphate in isolated mouse soleus muscle: concentration–response with BRL37344 (A); CL316,243 (B); SB226552 (C) and CGP12177 (D). The amount of [^{14}C]2-deoxyglucose-6-phosphate in digested muscle following incubation with agonist was measured by Ba_2SO_4 precipitation after a 45-min incubation with 5.5 mM 2-deoxyglucose containing a trace of [^{14}C]2-deoxyglucose. Significance levels are $^{*}P < 0.01$, $^{**}P < 0.001$ for a given point compared with the absence of agonist.

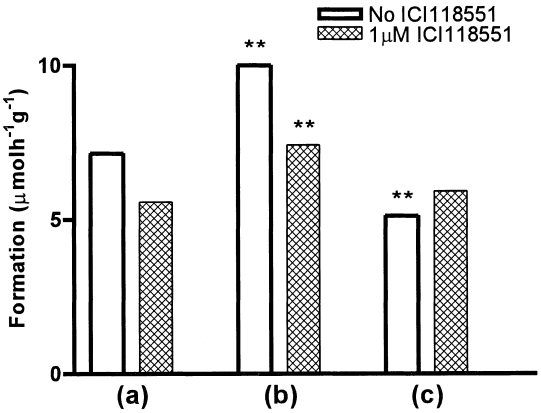

Fig. 2. Formation of 2-deoxyglucose-6-phosphate by isolated mouse soleus muscle: effects of ICI118551. Conditions of incubation are as follows: (a) no agonist present; (b) 10^{-10} M BRL37344; (c) 10^{-8} M BRL37344. Significance levels are $^{**}P < 0.001$ for a given point compared with the absence of BRL37344 at a constant concentration of ICI118551.

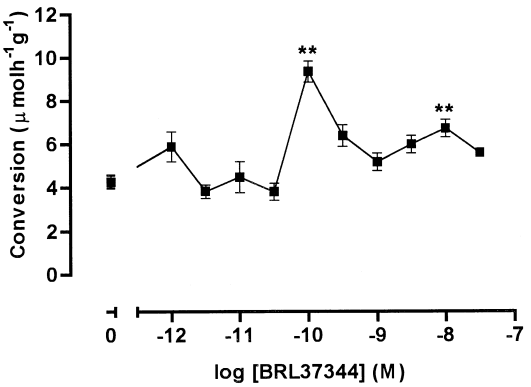

Fig. 3. Conversion of [2-^{14}C]pyruvate to $^{14}CO_2$ by isolated mouse soleus muscle: concentration–response with BRL37344. Radiolabelled $^{14}CO_2$ was collected following a 90-min incubation of isolated soleus muscle with 1 mM pyruvate containing a trace of [2-^{14}C]pyruvate. Significance levels are $^{**}P < 0.001$ relative to control (in the absence of BRL37344).

comparisons were made by using a one-way ANOVA followed by Dunnett's multiple comparison test to identify significance differences between groups.

3. Results

2-Deoxyglucose is transported and phosphorylated in a similar manner to glucose, but is not metabolised further. Fig. 1A shows the concentration response profile for the formation of 2-deoxyglucose-6-phosphate by mouse soleus muscle with BRL37344. A peak of formation of 2-deoxyglucose-6-phosphate was reached at 1×10^{-10} M BRL37344 (140% of basal values). At higher concentrations of BRL37344 (1×10^{-8} M), rates of formation of 2-deoxyglucose-6-phosphate were below basal values (58% of basal). This reduction of the rate of formation of 2-deoxyglucose-6-phosphate by 1×10^{-8} M BRL37344 (but not the increase by 1×10^{-10} M BRL37344) was blocked by the β_2-adrenoceptor antagonist, ICI118551 (Fig. 2).

Other agonists of the adipocyte β_3-adrenoceptor also stimulated muscle glucose uptake. The specific β_3-adrenoceptor agonist, CL316,243 (Bloom et al., 1992), caused a gradual increase in the rates of formation of 2-deoxyglucose-6-phosphate (Fig. 1B), achieving a 36% stimulation at 1×10^{-7} M. In a similar manner, SB226552, an agonist of the human β_3-adrenoceptor (Sennitt et al., 1998), caused a 54% stimulation at 1×10^{-9} M (Fig. 1C). Neither CL316,243 nor SB226552 caused rates of formation of 2-deoxyglucose-6-phosphate to fall below basal values at higher concentrations although there was a tendency for rates to decrease after reaching a peak. Fig. 1D shows the effects of CGP12177A on 2-deoxyglucose-6-phosphate-formation by mouse soleus muscle. This compound, which is a potent β_1- and β_2-adrenoceptor antagonist, is known to be a partial agonist of the adipocyte β_3-adrenoceptor (Van Liefde et al., 1993). At no concentration of CGP12177A tested (1×10^{-10} to 1×10^{-6} M) did stimulation of 2-deoxyglucose uptake occur but at higher concentrations (1×10^{-7} M), inhibition was apparent (to 86% of basal values).

The effect of BRL37344 on rates of oxidation of glucose, pyruvate and palmitate is shown in Table 1. BRL37344 was present at a concentration (1×10^{-10} M) that increased 2-deoxyglucose uptake and at 1×10^{-8} M, at which 2-deoxyglucose uptake is below basal. At 1×10^{-10} M, BRL37344 increased [U-^{14}C]glucose conversion

Table 1
Conversion of [U-^{14}C]glucose, [2-^{14}C]pyruvate, [1-^{14}C]pyruvate and [1-^{14}C]palmitate to $^{14}CO_2$ by isolated mouse soleus muscle

Addition	Conversion of substrate to $^{14}CO_2$ (μmol h^{-1} g^{-1})			
	[U-^{14}C]glucose	[2-^{14}C]pyruvate	[1-^{14}C]pyruvate	[1-^{14}C]palmitate
None	2.67 ± 0.2	4.28 ± 0.3	8.13 ± 1.2	0.195 ± 0.02
1×10^{-10} M BRL37344	4.00 ± 0.6^a	9.35 ± 0.5^b	9.20 ± 1.9	0.327 ± 0.03^b
1×10^{-8} M BRL37344	3.53 ± 0.5^a	6.73 ± 0.4^b	8.64 ± 1.7	0.278 ± 0.03^c

$^{14}CO_2$ from labelled substrates was collected and radioactivity determined.
[a] Statistical significance relative to control incubations containing no agonist at $P < 0.05$.
[b] Statistical significance relative to control incubations containing no agonist at $P < 0.001$.
[c] Statistical significance relative to control incubations containing no agonist at $P < 0.01$.

to $^{14}CO_2$ by 50%, increased [2-^{14}C]pyruvate conversion to $^{14}CO_2$ by 118% and [1-^{14}C]palmitate conversion to $^{14}CO_2$ by 68%. In contrast to the oxidation of [2-^{14}C]pyruvate, that of [1-^{14}C]pyruvate was not affected by 1×10^{-10} M BRL37344. Higher concentrations of BRL37344 did not reduce the rates of conversion of [U-^{14}C]glucose, [2-^{14}C]pyruvate or [1-^{14}C]palmitate to $^{14}CO_2$ to below the basal rates. However, all rates of oxidation reached a peak (at around 10^{-10} M) and declined at higher concentrations.

The full concentration–response curve of [2-^{14}C]pyruvate conversion to $^{14}CO_2$ with BRL37344 is shown in Fig. 3. A peak was reached at 10^{-10} M BRL37344, which represented a greater than 2-fold increase over basal values. Rates decreased at higher concentrations but, at 1×10^{-8} M, were still significantly above basal values (1.5-fold, Fig. 3). The addition of 1 μM propranolol (an antagonist of both the β_1- and β_2-adrenoceptor) did not alter the stimulation of [2-^{14}C]pyruvate oxidation elicited by 1×10^{-10} M BRL37344 (the rate was 9.57 μmol h^{-1} g^{-1} in the presence of 1 μM propranolol compared with 10.0 μmol h^{-1} g^{-1} in its absence).

In order to examine the possible intracellular signalling mechanism of the stimulatory effect of BRL37344 (1×10^{-10} M) on 2-deoxyglucose uptake and phosphorylation, two protein kinase inhibitors were used. At 200 nM, H-89, an inhibitor of protein kinase A ($K_i = 48$ nM) which shows some protein kinase C inhibitory properties at higher concentrations ($K_i = 32$ μM) (Findik et al., 1995), had no effect on the percentage stimulation of 2-deoxyglucose uptake and phosphorylation by 1×10^{-10} M BRL37344 (Fig. 4). However, H-89 did reduce both the basal rate (from 7.15 to 6.36 μmol h^{-1} g^{-1}) as well as the rate in the presence of 1×10^{-10} M BRL37344 (from 10.0 to 8.29 μmol h^{-1} g^{-1}). By contrast, bisindolylmaleimide IX, at 20 μM, which is a selective inhibitor of protein kinase C and has been used previously in a similar isolated incubated muscle system (Avignon et al., 1996), caused a negation of the BRL37344-induced stimulation of 2-deoxyglucose uptake and phosphorylation such that rates were reduced to slightly below basal values (to 87% of basal). Bisindolylmaleimide IX had no significant effect on basal rates of 2-deoxyglucose uptake and phosphorylation.

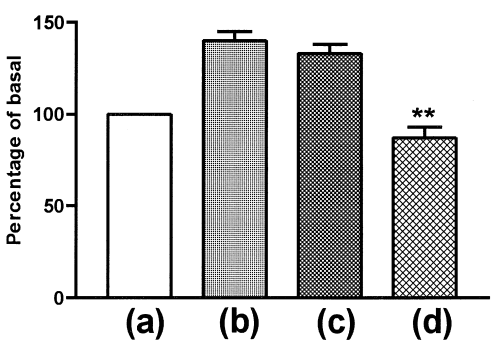

Fig. 4. Effects of H-89 and bisindolylmaleimide IX on the BRL37344-induced increase in 2-deoxyglucose formation. Isolated soleus muscles were incubated under the following conditions: (a) no addition, (b) 1×10^{-10} M BRL37344, (c) 1×10^{-10} M BRL37344 plus 2×10^{-7} M H-89, (d) 1×10^{-10} M BRL37344 plus 2×10^{-5} M bisindolylmaleimide IX. The amount of [^{14}C]2-deoxyglucose-6-phosphate in digested muscle following incubation for 45 min with 5.5 mM 2-deoxyglucose containing a trace of [^{14}C]2-deoxyglucose was measured using the Ba_2SO_4 precipitation procedure. Data are presented as a percentage of the corresponding value in the absence of BRL37344. Actual rates are given in the text. Statistical comparison: $^{**}P < 0.001$ is relative to the effect of 10^{-10} M BRL37344 alone (condition b).

4. Discussion

Previous studies on the effects of BRL37344 (or its methyl ester BRL35135) on glucose utilisation by rat tissue in vivo found that glucose uptake was increased in skeletal muscle independently of changes in plasma insulin (Abe et al., 1993; Liu and Stock, 1995). Furthermore, in vitro studies have shown that the effects were independent of changes in blood flow or hormonal responses caused by this β_3-adrenoceptor agonist. Thus, Liu et al. (1996) showed that BRL37344 was a very potent agonist of in vitro glucose utilisation in both soleus and extensor digitorum longus muscle preparations with peak effects occurring at concentrations as low as 1×10^{-11} M.

Studies on soleus muscles from rats have used either the complete muscle from young rats aged 21–31 days (Liu et al., 1996) or a stripped soleus muscle (Challis et al., 1988). Although the latter preparation can use tissue from adult rats, it involves significant tissue trauma. Since our ultimate aim is to examine genetic and dietary effects on the responses of BRL37344 and other agonists on muscle metabolism, we undertook studies using the adult mouse soleus preparation.

The rat studies performed by Liu et al. (1996) showed a peak in the concentration–response curve for BRL37344 at 1×10^{-11} M. In the present studies (Fig. 1A), the maximum stimulatory effect on 2-deoxyglucose uptake and phosphorylation was at 1×10^{-10} M (similar to that observed by Abe et al., 1993), a concentration of approximately 5×10^{-11} M producing half-maximal stimulation. This compares with the nanomolar EC_{50} values for the activation of lipolysis in brown and white adipose tissue from rats and mice (Arch et al., 1984; Wilson et al., 1984). These latter responses are thought to be mediated predominantly by the β_3-adrenoceptor. We have examined the possibility that the current effects on glucose uptake are mediated by an established β-adrenoceptor. The obvious possibility is a β_3-adrenoceptor with the difference in potency of BRL37344 in muscle and adipocytes arising through the muscle receptor being more tightly coupled to post-receptor mechanisms, having unusual pharmacology (akin to the findings of Konkar et al., 2000 that the β_4-adrenoceptor is really the β_1-adrenoceptor) or there being a difference in the number of spare receptors (Roberts et al., 1993; Sillence et al., 1993). However, we

believe it is more likely that the muscle effects are mediated by an, as yet, uncharacterised receptor that responds to BRL37344. This is supported by the failure of many groups to detect unequivocally β_3-adrenoceptor mRNA in skeletal muscle (Evans et al., 1996; Thomas and Liggett, 1993; Berkowitz et al., 1995), although, in contrast, Chamberlain et al. (1999) obtained an antibody raised against the cloned human β_3-adrenoceptor which detected an apparent β_3-adrenoceptor (or a protein with a similar epitope) in human skeletal muscle. The present study suggests two further pieces of evidence to support the lack of involvement of a β_3-adrenoceptor. Firstly, it is shown that CGP12177, which is a β_1/β_2-adrenoceptor antagonist and which stimulates lipolysis in mouse and rat adipocytes through the β_3-adrenoceptor, was inactive in stimulating the skeletal muscle receptor. Secondly, it is shown that the inhibitor of protein kinase A, H-89, which inhibits BRL37344-induced lipolysis (Murphy et al., 1993), failed to inhibit the BRL37344-stimulation of formation of 2-deoxyglucose-6-phosphate by soleus muscle. In contrast, the protein kinase C inhibitor, bisindolymaleimide IX, negated the stimulatory effect of BRL37344. The lack of involvement of protein kinase A in the muscle response is also supported by the studies of Roberts and Summers (1998) who could find no effect of β_3-adrenoceptor agonists, including BRL37344, on cyclic AMP accumulation in rat soleus muscle.

The failure of CGP12177 to stimulate muscle 2-deoxyglucose uptake and phosphorylation also rules out an involvement of the putative β_4-adrenoceptor (Galitzky et al., 1997; Kaumann, 1997). Previous pharmacological studies have suggested that cardiostimulant effects of CGP12177 were mediated by a β_4-adrenoceptor. Furthermore, there is evidence for β_4-adrenoceptor activity in β_3-adrenoceptor knock-out mice in both white and brown adipose tissue (Preitner et al., 1998) based on the stimulatory effect of CGP12177 and the inactivity of CL316,243. In the present study, both CL316,243 and the β_3-adrenoceptor agonist SB226552, which has weak β_1/β_2-adrenoceptor antagonist effects at the human receptors, were effective in stimulating the formation of 2-deoxyglucose-6-phosphate uptake. However, the effective concentration of CL316,243 was 1×10^{-7} M, which is a 100 times higher concentration than that found to be effective on the soleus muscle of young rats (Liu et al., 1996).

It also seems unlikely that BRL37344 at 1×10^{-10} M is acting at a β_2-adrenoceptor. BRL37344 is approximately 20-fold more selective for the β_3-adrenoceptor relative to the β_2-adrenoceptor and stimulatory concentrations at the β_2-adrenoceptor are in the range 5×10^{-7} to 1×10^{-8} M.

The concentration–response curve of BRL37344 on 2-deoxyglucose-6-phosphate formation is complex (Fig. 1A). The peak in formation of 2-deoxyglucose-6-phosphate at 1×10^{-10} M BRL37344 (which is not blocked by either 1 μM propranolol or 1 μM ICI118551) is followed by a marked trough at 1×10^{-8} M. In agreement with Liu et al. (1996), we consider that the latter effect is due to activity of the β_2-adrenceptor, since it is blocked by the β_2-adrenoceptor antagonist ICI118551 (Fig. 2). Furthermore, neither CL316,243 (which has virtually no β_2-adrenoceptor agonist activity) nor SB226552 (which has some β_2-adrenoceptor antagonist properties) caused rates of formation of 2-deoxyglucose-6-phosphate to fall below basal.

Previous studies in isolated rat soleus muscles have only examined the effect of BRL37344 on glucose uptake and phosphorylation and glycogen synthesis. In the present study, we have confirmed the stimulatory effect of BRL37344 at low concentrations on 2-deoxyglucose uptake and phosphorylation. Our experiments, as previously (Liu et al., 1996), were undertaken with a very low insulin concentration sufficient only to maintain metabolic function. At this low insulin concentration, the measured rate of 2-deoxyglucose-6-phosphate formation is considered representative of glucose uptake predominantly via the GLUT-1 glucose transporter isoform. In the present study, we also show for the first time that BRL37344, at the low concentration of 1×10^{-10} M, also stimulates the rates of glucose oxidation (50% increase) and palmitate oxidation (70% increase). In addition, the oxidation of [2-^{14}C]pyruvate is stimulated more than 2-fold but there is little change in rates of oxidation of [1-^{14}C]pyruvate. The rate of conversion of [2-^{14}C]pyruvate to $^{14}CO_2$ represents rates of oxidative reactions of the tricarboxylic acid cycle. By contrast, the rate of conversion of [1-^{14}C]pyruvate to $^{14}CO_2$ has often been used as a measure of intracellular pyruvate dehydrogenase activity. Thus, our findings indicate that the low concentrations of BRL37344 do not stimulate pyruvate dehydrogenase activity in the soleus muscle. However, $^{14}CO_2$ can also be produced from [1-^{14}C]pyruvate by reactions of the tricarboxylic acid cycle if pyruvate carboxylase is active as a means of introducing pyruvate carbon into the tricarboxylic acid cycle. Thus, the ratio of ($^{14}CO_2$-production from [2-] or [3-^{14}C]pyruvate):($^{14}CO_2$-production from [1-^{14}C]pyruvate) has been used to indicate the importance of the anaplerotic activity of pyruvate carboxylase relative to that of pyruvate dehydrogenase (Curi et al., 1988), as a means of introducing pyruvate carbon into the tricarboxylic acid cycle. At 1×10^{-10} M BRL37344, the ratio increased from 0.5 to 1.0 and decreased to 0.8 at higher concentrations of BRL37344. This suggests that pyruvate carboxylase has a significant role in the increased rate of oxidation of pyruvate under conditions where β_{skel}-adrenoceptor is stimulated and that this reflects increased tricarboxylic acid cycle activity. It seems likely that the increase in pyruvate oxidation is a consequence of providing oxaloacetate to support increased tricarboxylic acid cycle activity associated with increased fatty acid oxidation. These findings may indicate a separation of the anabolic, insulin-stimulated pyruvate dehydrogenase pathway from the catabolic pathway using pyruvate carboxylase, which is stimulated by BRL37344 acting via

the β_{skel}-adrenoceptor. Earlier studies by Taneshita et al. (1997) showed that BRL37344 and insulin increased glucose transport in myotubules via different mechanisms and resulted in additive effects.

Treatment of whole animals with BRL37344 resulted in increased energy expenditure (Arch et al., 1984). It has generally been believed that such action was predominantly the result of thermogenic activity in brown adipose tissue. The present findings, together with earlier results of Liu and Stock (1995) and Liu et al. (1996), suggest that some of the increase in whole body energy expenditure caused by BRL37344 may be occurring in skeletal muscle via concurrent activation of the β_{skel}-adrenoceptor. Such an effect may explain some of the previously recorded results of treating whole animals. For example, Thurlby and Ellis (1986) observed that, in contrast to noradrenaline, much of the increase in oxygen consumption caused by the β_3-adrenoceptor agonist BRL28410 occurred in skeletal muscle rather than brown adipose tissue.

The thermogenic effects of BRL35135 in brown adipose tissue require the presence of uncoupling protein-1 (UCP-1) as a proton leak that allows oxidative metabolism to occur without the production of ATP (Nicholls et al., 1984). Agonists of the β_3-adrenoceptor also induce transcription of the UCP-1 gene in brown adipocytes within 15 min of exposure (Ricquier et al., 1986). The recent discovery of UCP-3, which is expressed predominantly in skeletal muscle and brown adipose tissue (Boss et al., 1997; Vidal-Puig et al., 1997), has raised the possibility that UCP-3 may have a role in thermogenesis in muscle (Gong et al., 1997). Indeed β_3-adrenoceptor agonists also increased UCP-3 mRNA expression in skeletal muscle, as did exercise training, whereas denervation caused down-regulation (Tsuboyama-Kasaoka et al., 1998). The possibility that such thermogenic responses are mediated by an, as yet, uncharacterised receptor rather than the β_3-adrenoceptor is supported by the recent data of Boss et al. (1999). They showed that treatment of β_3-adrenoceptor knockout mice with BRL37344 resulted in an increase in both UCP-2 and UCP-3 mRNAs in skeletal muscle. However, they speculated that the effect was mediated by a β_4-adrenoceptor. On the basis of the current work, we believe that this response is more likely to be mediated by the β_{skel}-adrenoceptor.

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