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European Journal of Pharmacology 295 (1996) 199–205

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The acid metabolite of ZD7114 is a partial agonist of lipolysis mediated by the rat β_3-adrenoceptor

Rachel M. Mayers *, Simon P. Quayle, Andrew J. Thompson, Thomas L. Grant, Brian R. Holloway

Cardiovascular and Metabolism Research Department, Zeneca Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK

Received 1 June 1995; revised 29 September 1995; accepted 10 October 1995

Abstract

Experiments were performed to characterise the lipolytic effects of the acid metabolite, ZM215001, ((*S*)-4-[2-hydroxy-3-phenoxy-propylamino-ethoxy] phenoxyacetic acid) of the putative β_3-adrenoceptor agonist, ZD7114 ((*S*)-4-[2-hydroxy-3-phenoxy-propylamino-ethoxy]-*N*-(2-methoxyethyl) phenoxyacetamide) on isolated rat white adipocytes. ZM215001 was used for these studies since it is the predominant moiety after in vivo administration of ZD7114. The agonist properties of ZM215001 were assessed in comparison to the standard nonselective β-adrenoceptor agonist (±)-isoprenaline and the β_3-adrenoceptor-selective agonist BRL 37344. Isoprenaline, BRL 37344 and ZM215001 all stimulated the rate of free fatty acid release from isolated adipocytes with the order of potency being BRL > isoprenaline > ZM215001. The maximum effect of BRL 37344 was equivalent to that of isoprenaline, but ZM215001 achieved only 30% of the maximum isoprenaline response. ZM215001 competitively antagonised the lipolytic response to BRL 37344 ($pA_2 = 7.26$), whereas the agonist effects of BRL 37344 were not antagonised competitively by the selective antagonists ICI 118551 and CGP 20712A, at concentrations which would be expected to block β_1- and β_2-adrenoceptors respectively. These results indicate that ZM215001 has low intrinsic activity at the rat adipocyte β_3-adrenoceptor, and is a partial agonist of lipolysis in rat white adipocytes.

Keywords: Adipocyte, rat, white; Lipolysis; β_3-Adrenoceptor; ZD7114, partial agonist effect

1. Introduction

It is now well established that the principal adrenoceptor responsible for the stimulation of lipolysis in rat adipocytes is the atypical or β_3-adrenoceptor. Selective agonists for this receptor, notably BRL 26830A, BRL 35135A and their respective acid metabolites BRL 28410 and BRL 37344, were first described by Arch et al. (1984). A distinguishing characteristic of the β_3-adrenoceptor is that the selective agonist BRL 37344 (pD_2 7.5–8.7, Arch and Kaumann, 1993) is more potent at this receptor than are the nonselective agonists noradrenaline (pD_2 6.9) and isoprenaline (pD_2 6.9–7.7). In addition, isoprenaline-stimulated lipolysis in rat adipocytes is resistant to blockade by propranolol, requiring approximately 0.3 μM propranolol to induce a 2-fold shift of the concentration-response curve (pA_2 value 6.5–6.8). In systems mediated by β_1-adrenoceptors (rat or guinea pig atrial rate) or by β_2-adrenoceptors (relaxation of guinea pig trachea or rat diaphragm contraction) isoprenaline is more potent than BRL 37344. In addition, the agonist effects of isoprenaline are antagonised by low concentrations of propranolol (pA_2 values 7.9–8.7, Arch and Kaumann, 1993). Further evidence for a third type of β-adrenoceptor was provided by Emorine et al. (1989) who isolated a gene which coded for a third β-adrenoceptor from a human genomic library. When expressed in Chinese hamster ovary (CHO) cells this receptor displayed many of the characteristics of the β-adrenoceptor which mediates lipolysis in rat adipose tissue, being resistant to propranolol blockade and capable of being activated by BRL 37344. Both rat (Granneman et al., 1991) and mouse (Nahmias et al., 1991) β_3-adrenoceptor genes have subsequently been cloned and expressed, exhibiting a similar pharmacological profile.

* Corresponding author. Tel.: (44) 01625 512371; fax: (44) 01625 586707.

SSDI 0014-2999(95)00662-1

Analysis of a range of rat tissues indicates that this receptor is expressed in abundance only in adipose tissue (Granneman et al., 1991). However, in this study and that of Emorine et al. (1989), low levels were detected in rat ileum, consistent with the observations in various laboratories (Bond and Clarke, 1988; Macdonald and Lamont, 1992) of a functional β_3-adrenoceptor in rodent gastrointestinal smooth muscle.

Attempts to identify the receptor in human tissue have yielded variable results. Thomas and Liggett (1993), investigating a large number of tissues from one subject, failed to detect mRNA for the β_3-adrenoceptor. However, in the same year four independent groups reported the detection of β_3-adrenoceptor mRNA in white adipose tissue after PCR amplification (Granneman et al., 1993; Lönnqvist et al., 1993; Revelli et al., 1993; Krief et al., 1993). Granneman et al. (1993) and Krief et al. (1993) also found β_3-adrenoceptor expression in human colon, the latter group also detecting a surprisingly high level of expression in gall bladder. Evidence for functional β_3-adrenoceptors in human adipose tissue has also been difficult to obtain: Langin et al. (1991) failed to detect significant stimulation of lipolysis in human adipocytes by the selective agonist BRL 37344, whereas Hollenga et al. (1990) found the compound had an effect which was only 18% of the isoprenaline response. However, Lönnqvist et al. (1993), using a highly sensitive glycerol assay and a low cell number, were recently able to demonstrate stimulation of lipolysis by this compound up to 66% of the maximum effect of isoprenaline.

Zeneca ZD7114 ((*S*)-4-[2-hydroxy-3-phenoxy-propylamino-ethoxy]-*N*-(2-methoxyethyl) phenoxyacetamide) was developed as an agonist at the β_3-adrenoceptor, for use in the treatment of obesity and non-insulin-dependent diabetes. In vivo, ZD7114 stimulates oxygen consumption in conscious rats, at doses which have minimal direct effects on cardiovascular parameters (Holloway et al., 1991a, b). The increase in oxygen consumption was associated with an increase in the activity of brown adipose tissue, indicated by an increase in specific GDP binding to brown adipose tissue mitochondria. The maximum effect of ZD7114 on both oxygen consumption and brown adipose tissue activity was indistinguishable from the maximum effect achieved by BRL 35135 in the same experimental models (Mayers, unpublished results).

When administered to normal, lean, human volunteers, 150 mg per day ZD7114 elevated basal metabolic rate by 3.6% (Goldberg et al., 1992), and in obese patients treated with 100 mg per day ZD7114, a 1% increase in 24-h energy expenditure was observed (Toubro et al., 1993). However, obese patients who had received 400 mg BRL 26830A for 18 weeks exhibited a significant, 12% increase in the energy expenditure induced by a subsequent acute dose of the same compound, compared to patients treated with placebo for the same period (Connacher et al., 1988). BRL 35135 dosed at 0.1 mg/kg also stimulated energy expenditure by 16% in lean volunteers (Smith et al., 1989). ZD7114 appears to be less efficacious in man than would be predicted from energy expenditure experiments in rats.

The experiments described in the present study were carried out in order to investigate the potency and intrinsic activity of ZD7114 at the rat adipocyte β_3-adrenoceptor mediating lipolysis. The principal metabolite of ZD7114 after in vivo administration is the related acid ZM215001 ((*S*)-4-[2-hydroxy-3-phenoxy-propylamino-ethoxy] phenoxyacetic acid) (unpublished results), and this compound was used for the in vitro studies described.

A preliminary report of these results has been given (Quayle et al., 1993).

2. Materials and methods

2.1. Preparation of adipocytes

Epididymal adipocytes from male Wistar rats (170–200 g) were isolated by slight modification of the method of Wilson et al. (1984). Tissue was digested with 1.5 mg/ml collagenase (type II) in Krebs-Ringer bicarbonate buffer (KRB), pH 7.4, composed of (mM): NaCl 118.5, KCl 4.7, $MgSO_4$ 1.18, $CaCl_2$ 1.26, KH_2PO_4 1.19, $NaHCO_3$ 25, pregassed with 5% CO_2 in O_2. Digestion was carried out for 105 min, then cells were separated by flotation and washed 4 times with 20 ml fresh KRB. A fifth and final wash was carried out with KRB containing 2% bovine serum albumin (KRB/BSA).

2.2. Preparation of compounds

Solutions of compounds were prepared at 10 times the required final concentration in KRB/BSA containing 1 mg/ml ascorbic acid to prevent oxidation. Initial solutions of BRL 37344 and ZM215001 were prepared at 0.1 M in dimethyl sulphoxide (DMSO), and diluted as appropriate.

2.3. Agonist experiments

300000 cells were incubated in the presence or absence of agonist in a total volume of 1 ml KRB/BSA, under an atmosphere of 5% CO_2, 95% O_2. Incubations were carried out in polyethylene vials, in a shaking water bath at 37°C. After 90 min, the vials were removed to ice and cells allowed to rise to the surface of the reaction medium for 10 min. An aliquot of the infranatant was removed and assayed for free fatty acids or glycerol. Preliminary experiments demon-

strated that free fatty acid release is linear with time over this period up to a maximum concentration of 2.4 mEq/l at which feedback inhibition of lipolysis occurs.

Agonist concentration-response curves were constructed to ZM215001, BRL 37344 and isoprenaline.

2.4. Antagonist experiments

Experiments with antagonists were carried out as described above except that cells were pre-incubated in KRB/BSA for 30 min in the presence or absence of antagonist before the addition of agonist. Incubations were continued for 90 min after the addition of the agonist. In each experiment separate incubations containing isoprenaline alone at 3 μM were included to determine the maximum rate of lipolysis.

For antagonist experiments, concentration-response curves were constructed to BRL 37344 in the presence or absence of several concentrations of either ZM215001, ICI 118551 or CGP 20712A.

2.5. Estimation of free fatty acids

The concentration of free fatty acids in an aliquot of the infranatant was estimated using a WAKO NEFA-C assay kit. This method is based on the acylation of coenzyme-A by acyl-CoA synthetase. The acyl-CoA produced is oxidised, producing hydrogen peroxide which in the presence of added peroxidase allows the oxidative condensation of 3-methyl-*N*-ethyl-*N*-(β-hydroxyethyl)-aniline with 4-aminoantipyrine to form a purple coloured product. The recommended procedure for this kit was slightly modified to enable the assay to be carried out in 96-well plates. 50 μl of the sample, diluted 3-fold in KRB/BSA, was mixed with 100 μl of reagent A (dissolved in 6.7 ml solvent A) and incubated at 37°C for 10 min. 50 μl reagent B (dissolved in 7.5 ml solvent B) was added and incubated for a further 10 min before reading the optical density at 550 nm.

2.6. Estimation of glycerol

The concentration of glycerol in the infranatant was estimated using the glycerol phosphate oxidase-Trinder method (Trinder, 1969; Barham and Trinder, 1972) using a triglyceride (GPO-Trinder) assay kit. A 40 μl aliquot of the infranatant was first depleted of ascorbic acid by incubation with 3 U/ml ascorbate oxidase, in a total volume of 50 μl, for 20 min at 37°C. 200 μl GPO-Trinder reagent was added and after a further 10 min at 37°C, the optical density determined at 550 nm. Unknowns were compared to a commercially available glycerol standard, diluted in 0.1 mg/ml ascorbic acid.

2.7. Data analysis

All concentration-response curves were expressed relative to the isoprenaline response at 3 μM, a concentration which gives the maximum increase in rate of lipolysis. pK_B values were derived from the ZM215001 data according to the method of Kaumann and Blinks (1980). For comparison, the same data were analysed by the method of Arunlakshana and Schild (1959). Where only a single concentration of antagonist was effective, apparent pA_2 values were derived by the concentration-ratio method of Furchgott (1972). Unless otherwise indicated, results are expressed as the means ± S.E.M. of three experiments.

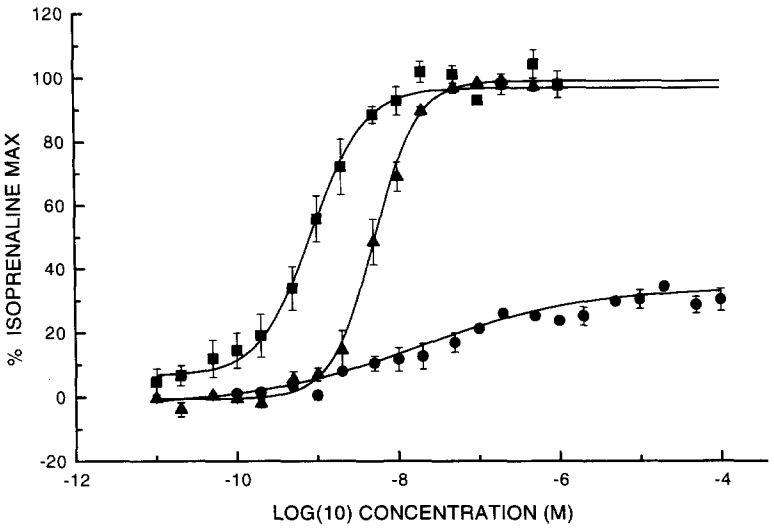

Fig. 1. Effects of β_3-adrenoceptor agonists on lipolysis in rat epididymal adipocytes. Concentration-response curves to (▲) (±)-isoprenaline, (■) BRL 37344 and (●) ZM215001. Each point represents the percentage of the response to 3 μM isoprenaline. Data shown are the mean ± S.E.M. of three separate experiments.

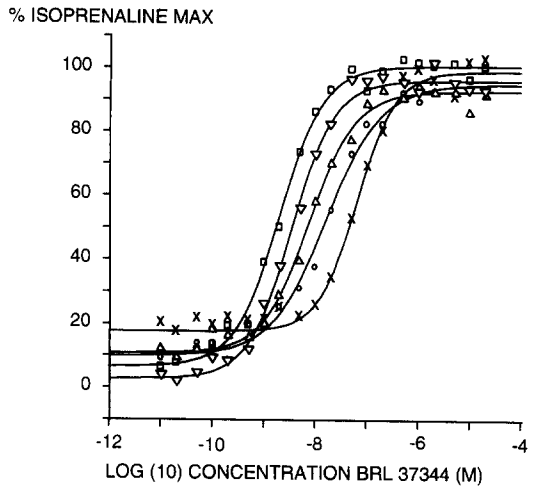

Fig. 2. Antagonism of lipolysis induced by BRL 37344 by ZM215001. Control (■); ZM215001 0.03 μM (▼), 0.1 μM (▲), 0.3 μM (●) or 1.0 μM (×). Each point represents the percentage of the response to 3 μM isoprenaline and is the mean of two separate determinations. Data shown are from a representative experiment.

2.8. Materials

Bovine serum albumin (essentially fatty acid-free), collagenase (type II, 475 U/mg), (±)-isoprenaline hemisulphate, ascorbate oxidase, glycerol standard and triglyceride (GPO-Trinder) kit were obtained from Sigma (Poole, UK). Buffer salts were obtained from BDH (Poole, UK) and the WAKO NEFA-C kit from Alpha Laboratories. All other compounds were synthesised at Zeneca Pharmaceuticals (Macclesfield, UK).

3. Results

3.1. Agonist activity

Isoprenaline, BRL 37344 and ZM215001 (the principal metabolite of ZD7114), all caused a concentration-related increase in the rate of lipolysis by isolated rat epididymal adipocytes (Fig. 1). In a total of 12 experiments, the basal level of free fatty acid release measured in the absence of agonists was 0.34 ± 0.08 mEq/l/90 min. The inclusion of 3 μM isoprenaline in the incubation medium resulted in an approximately 6-fold increase in the rate of free fatty acid release to 1.95 ± 0.34 mEq/l/90 min. Fig. 1 summarises the results from three experiments in which the agonist effects of isoprenaline, BRL 37344 and ZM215001 were compared. The mean half-maximum effect (pD_2) for isoprenaline was 8.13 ± 0.04. BRL 37344 (pD_2 8.85 ± 0.23) was approximately 6-fold more potent than isoprenaline. The maximum effect elicited by BRL 37344 was similar to that elicited by 3 μM isoprenaline (107.2 ± 2.1% of the isoprenaline response). In the same experiments, ZM215001 elicited a concentration-related increase in the rate of free fatty acid release from rat white adipocytes, with a pD_2 of 7.42 ± 0.27. However, the maximum response to ZM215001 was only 30.2 ± 2.7% of the maximum effect elicited by isoprenaline.

One possible explanation for the apparent submaximal rate of lipolysis in the presence of high concentrations of ZM215001 is that this compound causes an increased rate of re-esterification of free fatty acids. In

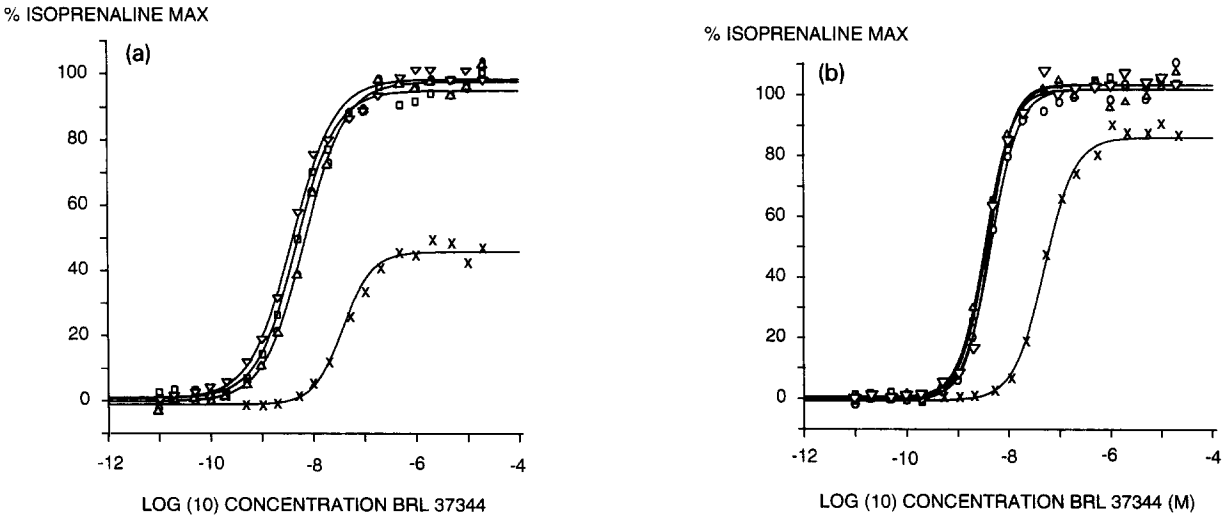

Fig. 3. Antagonism of lipolysis induced by BRL 37344 by (a) CGP 20712A. Control (■); CGP 20712A 0.1 μM (▼), 1.0 μM (▲), 10 μM (○) or 100 μM (×); and (b) ICI118551 at 0.03 μM (▼), 0.3 μM (▲), 3.0 μM (●) or 30 μM (×). Each point represents the percentage of the response to 3 μM isoprenaline and is the mean of two separate determinations. Data shown are from a representative experiment.

order to eliminate this possibility, samples from one experiment were assayed for both glycerol and free fatty acids. In this experiment, the maximum effect of ZM215001 was 35.5% of the maximum isoprenaline response when release of free fatty acid was used as an indicator of lipolysis, and when free glycerol was determined ZM215001 attained 36.2% of the maximum isoprenaline response.

3.2. Antagonist effect of ZM215001

ZM215001 was tested as a potential antagonist of the lipolytic response to BRL 37344. Preincubation of rat epididymal adipocytes with ZM215001 (30 nM–1 μM) prior to the addition of the agonist resulted in consistent rightward shifts of the concentration-response curve to BRL 37344 (Fig. 2). Analysis of the antagonist concentration-response curves gave a mean pK_B value for the four antagonist concentrations tested of 7.56 ± 0.09. A Schild plot derived from the same data gave a pA_2 value of 7.34 ± 0.32 ($n = 3$), with a slope which was not significantly different from unity (1.08 ± 0.14). The rate of lipolysis induced by ZM215001 in the absence of BRL 37344 increased with increasing concentration up to approximately 20% of the maximum isoprenaline response at 1 μM ZM215001: this was consistent with the agonist activity of ZM215001.

3.3. Antagonism of lipolysis by a β_1-adrenoceptor antagonist

In three experiments where adipocytes were preincubated with the selective β_1-adrenoceptor antagonist CGP 20712A, at concentrations ranging from 0.1 μM to 10 μM, prior to the addition of BRL 37344 in the range 0.01 nM to 10 μM, these concentrations of antagonist did not shift the concentration-response curve to BRL 37344 (Fig. 3a). However, in two of these experiments adipocytes were also treated with 100 μM CGP 20712A, which both shifted the concentration-response curve to the right (pD_2 7.45: individual values 7.47, 7.45) and depressed the maximum response to 47% (50, 43) of that elicited by BRL 37344 alone. From the rightward shift induced by this concentration, an apparent pA_2 value of 4.70 (4.56, 4.83) was estimated.

3.4. Antagonism of lipolysis by a β_2-adrenoceptor antagonist

Preincubation of adipocytes with the selective β_2-adrenoceptor antagonist ICI 118551 at 30 nM to 3 μM prior to the addition of BRL 37344 in the range 0.01 nM to 10 μM did not affect the concentration-response curve to BRL 37344. At 30 μM ICI 118551 both shifted the concentration-response curve to the right ($pD_2 = 7.16 \pm 0.18$, $n = 3$) and slightly depressed the maximum response to approximately 90% of that elicited by agonist alone. (Fig. 3b). The apparent pA_2 value estimated from this shift was 5.74 ± 0.09 ($n = 3$).

4. Discussion

ZM215001, administered at concentrations up to 100 μM, stimulated lipolysis in rat white adipocytes with a half-maximal effect at 38.0 nM (pD_2 7.42), thus it has lower potency as an agonist than BRL37344 (pD_2 8.85) and isoprenaline (pD_2 8.13). The potency of ZM215001 has not been investigated in models of β_1- or β_2-adrenoceptor agonist activity; however, the racemate ZM201651 stimulated the rate of contration of guinea pig right atria (β_1) and relaxation of tracheal chain (β_2) only at concentrations greater than 1 μM ($pD_2 < 5.0$) (Holloway et al., 1989). ZM215001 has been tested at a single concentration of 10 μM with similar results: in atria, 10 μM ZM215001 induced $18.5 \pm 2.5\%$ of the maximal response, and in trachea, $26.1 \pm 2.7\%$ of the maximal relaxation (Growcott et al., unpublished results). In similar preparations, BRL 37344 produced marked stimulation of atrial rate and relaxation of the tracheal chain at this concentration (Arch et al., 1984). Therefore the data suggest that the stimulation of lipolysis by ZM215001 is not mediated by β_1- or β_2-adrenoceptors and is consistent with activity at the β_3-adrenoceptor.

ZM215001 achieved a maximum increase in the rate of lipolysis that was only 30% of that seen with either isoprenaline or BRL 37344. It seemed possible that this result might be an artefact arising from our use of free fatty acid release as a measure of lipolysis, whereas most other workers have estimated the release of glycerol. Free fatty acids are potentially subject to reesterification whereas liberated glycerol cannot be metabolised since adipocytes are deficient in glycerol kinase (Steinberg et al., 1961). However, our results were similar whichever product of lipolysis was determined. It therefore appears that ZM215001 is a β_3-adrenoceptor agonist with low intrinsic activity in this model.

In contrast, BRL 37344 stimulated lipolysis in the same model, achieving a maximum effect equivalent to that of isoprenaline. BRL 37344 was 6-fold more potent than isoprenaline, which is consistent with the results of Hollenga et al. (1990, 1991) and Langin et al. (1991), who reported potency ratios of 11, 12, and 8, respectively. Furthermore, the agonist effect is resistant to blockade by CGP20712A, a selective β_1-adrenoceptor antagonist (Dooley et al., 1986). At concentrations of up to 10 μM, no shift was observed in the concentration-response curve to BRL 37344 whereas 100 μM CGP 20712A caused a rightward shift of the

concentration-response curve in conjunction with a depression of the maximum response. The resulting low pK_B value, together with the observation of a decreased maximum response, is consistent with noncompetitive inhibition of the response to BRL 37344. Similarly, the selective β_2-adrenoceptor antagonist ICI 118551 only antagonised the lipolytic effect of BRL 37344 at a relatively high concentration. We have therefore confirmed the results of Hollenga and Zaagsma (1989) that in rat white adipocytes, the activation of lipolysis by BRL 37344 is due entirely to effects at β_3-adrenoceptors and this compound has intrinsic activity which is equivalent to that of isoprenaline in this model.

ZM215001 antagonised the lipolytic response to BRL 37344 with a pK_B value of 7.56. This is comparable with the pK_B value obtained by Macdonald and Lamont (1992) for inhibition of isoprenaline-mediated relaxation of rat distal colon by ZD7114 in the presence of selective β_1- or β_2-adrenoceptor antagonists. However, Growcott et al. (1993) obtained a lower affinity ($pA_2 = 6.7$) than Macdonald and Lamont (1992) with ZD7114 against the BRL 37344-mediated relaxation of rat ileum. Propranolol, a nonselective β-adrenoceptor antagonist, inhibits the lipolysis induced by BRL 37344 ($pA_2 = 6.3$, Langin et al., 1991): ZM215001 is therefore a more potent antagonist at the rat β_3-adrenoceptor than propranolol and equivalent to bupranolol ($pA_2 = 7.3$, Langin et al., 1991) and *l*-alprenolol ($pA_2 = 7.35$, Hollenga et al., 1990).

We found that ZM215001 has only 30% of the intrinsic activity of isoprenaline as an agonist of lipolysis in isolated rat white adipocytes, whereas Holloway et al. (1991a) observed that ZM215001 stimulated respiration in adipocytes isolated from rat brown adipose tissue up to a maximum level equivalent to that evoked by isoprenaline. ZM215001 also had intrinsic activity equivalent to that of isoprenaline in lipolysis studies using rat brown adipocytes (Sudera and Stock, personal communication). Furthermore, ZD7114 apparently had full efficacy as a thermogenic agent in the rat in vivo, stimulating oxygen consumption and activating brown adipose tissue (as demonstrated by the increased binding of guanosine diphosphate (GDP) to isolated brown adipose tissue mitochondria) with maximum effects that were indistinguishable from the maximum effect elicited by BRL 35135 (Holloway et al., 1991b). It therefore appears that ZM215001 may be a full agonist of oxygen consumption and lipolysis at β_3-adrenoceptors in rat brown adipocytes while acting as a partial agonist of lipolysis at β_3-adrenoceptors in white adipose tissue. This may be indicative of a difference in receptor reserve between white and brown adipose tissue.

CGP 12177, a compound which was initially identified as a β_1- and β_2-adrenoceptor antagonist, and was recently demonstrated to have the properties of a β_3-adrenoceptor agonist, also has low intrinsic activity at stimulating lipolysis in rat white adipocytes (Langin et al., 1991). In these experiments CGP 12177 elicited 36% of the maximum isoprenaline response. The lipolytic effect of CGP 12177 has not been tested in brown adipocytes but the maximum stimulation of adenylate cyclase elicited by this compound was lower than that of isoprenaline (Granneman and Whitty, 1991). Moreover, it was 6-fold less potent at stimulating adenylate cyclase in white than in brown adipocytes, which may be an indication of there being a lower receptor reserve in rat white adipose tissue (Arch and Kaumann, 1993). The suggestion that brown adipose tissue may have a greater number of 'spare' β_3-adrenoceptors than white adipose tissue is further supported by the report that mRNA encoding for the β_3-adrenoceptor represents a greater proportion of total extracted mRNA in white adipose tissue than in brown adipose tissue (Emorine et al., 1989; Cousin et al., 1993). The difficulty that has been experienced in detecting the β_3-adrenoceptor in human adipose tissue suggests that it is in very low abundance in this species. This may account for the lack of effectiveness of some selective β_3-adrenoceptor agonists, including ZD7114, in man. Furthermore, the low intrinsic activity of ZM215001 as an agonist of lipolysis in rat white adipocytes is likely to be indicative of even lower intrinsic activity as a stimulant of lipolysis in human adipocytes.

In conclusion, we have demonstrated that the putative β_3-adrenoceptor agonist ZM215001, the active metabolite of ZD7114, has the characteristics of a partial agonist of lipolysis in rat white adipocytes.

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