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Direct evidence for the atypical nature of functional β-adrenoceptors in rat adipocytes

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1 The nature of the rat epididymal adipocyte β-adrenoceptor was investigated by studying the effects of β_1- and β_2-selective antagonists on lipolysis induced by (−)-isoprenaline and the lipolytically selective agonist BRL 37344.

2 From 10 nM to 10 μM, the potent and highly selective β_1-adrenoceptor antagonist CGP 20712A did not influence the concentration-response curve (CRC) of BRL 37344 whereas small but consistent shifts to the right of the (−)-isoprenaline-induced CRC were observed. Clear rightward shifts of the CRCs induced by both (−)-isoprenaline and BRL 37344 were produced only at 100 μM CGP 20712A with the corresponding pA_2 values being 4.80 and 4.61, respectively.

3 When the β_2-selective antagonist ICI 118,551 was used at 10 μM and higher, clear and concentration-dependent shifts to the right of the CRCs of both agonists were observed. The slopes of the Schild plots did not deviate significantly from unity, the pA_2 values being 5.49 and 5.33 against (−)-isoprenaline and BRL 37344, respectively.

4 The results demonstrate that (−)-isoprenaline-induced lipolysis in rat white adipocytes is mediated predominantly by atypical β-adrenoceptors, whereas the typical β_1-adrenoceptors play a small, subordinate role. The lipolytically selective agonist BRL 37344 acts solely through atypical β-adrenoceptors.

Introduction

The classification of the β-adrenoceptor mediating lipolysis in rat adipocytes has been the subject of much debate. Originally, Lands *et al.* (1967), using a series of catecholamine agonists, classified it as a β_1-adrenoceptor. However, Harms *et al.* (1974), and De Vente *et al.* (1980) suggested a hybrid or atypical type of β-adrenoceptor mediating lipolysis with low affinity for both β_1- and β_2-selective and non-selective antagonists. Moreover, fat cell β-adrenoceptors showed lower stereoselectivity ratios for antagonist enantiomers than typical β_1-(cardiac) and β_2-(skeletal muscle) adrenoceptors (Harms *et al.*, 1977).

Bojanic & Nahorski (1983) and Bojanic *et al.* (1985) more recently provided evidence for the co-existence of at least two subtypes of β-adrenoceptors on adipocyte membranes. In radioligand binding studies in which subnanomolar concentrations of [^{125}I]-cyanopindolol were used, they found predominantly typical β_1-adrenoceptors, while in functional experiments they found the β-adrenoceptor mediating adenylyl cyclase activation to be clearly atypical, resembling that of intact cells mediating lipolysis. This conclusion was strengthened by the observation that the irreversible photo-affinity β-antagonist *p*-aminobenzylcarazolol, in concentrations that almost completely inhibited adenylyl cyclase activation of rat reticulocyte membranes, was virtually ineffective in adipocyte membranes (Bojanic & Nahorski, 1984). Furthermore, the introduction of a novel series of β-adrenoceptor agonists that selectively stimulate lipolysis, provided important support for the concept of an atypical β-adrenoceptor mediating lipolysis (Arch *et al.*, 1984).

However, more recently Bahouth & Malbon (1988) disputed these claims. They studied the β_2-selective antagonist ICI 118,551 and the new 10,000-fold β_1-selective antagonist CGP 20712A (Dooley *et al.*, 1986) on lipolysis, adenosine 3:5-cyclic monophosphate (cyclic AMP) accumulation

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and radioligand binding displacement in rat adipocytes and membranes. The binding experiments confirmed that the adrenoceptor sites occupied by low concentrations of the radioligand are predominantly of the β_1-type. Since their functional studies also showed that CGP 20712A was a more effective antagonist than ICI 118,551 it was concluded that the character of the rat fat cell β-adrenoceptor is solely β_1. However, close inspection of the functional data revealed that the potency difference between the two antagonists did not agree at all with the binding affinities for a typical β_1-adrenoceptor. Therefore we decided to investigate in more detail CGP 20712A and ICI 118,551 for antagonism of lipolysis induced by isoprenaline and by the lipolytically selective β-adrenoceptor agonist BRL 37344. The results show unequivocally that a small part of the lipolysis mediated by isoprenaline is through its action at typical β_1-adrenoceptors, but the major part is through atypical β-adrenoceptors, while the lipolytically selective compound BRL 37344 acts solely through the atypical β-adrenoceptor.

Methods

Epididymal adipocytes from male Wistar rats (210–230 g) were isolated essentially according to Rodbell (1964). The rats were killed by a blow on the head. Fat pads were removed and placed in Krebs-Henseleit buffer composed of (mM): NaCl 117.5, KCl 5.6, $MgSO_4$ 1.18, $CaCl_2$ 2.52, $NaHPO_4$ 1.28, $NaHCO_3$ 25.0 and glucose 5.5, at room temperature, pregassed with 5% CO_2 in O_2, pH 7.4. After removal of the major blood vessels the fat pads were chopped in 600 μm slices with a McIlwain tissue chopper. Cells were then isolated by incubation in a shaking waterbath, in a Teflon vessel containing 3 ml Krebs-Henseleit solution per fat pad, 0.33 mg ml^{-1} collagenase and 1% bovine serum albumin, at 37°C under an atmosphere of 5% CO_2 in O_2, pH 7.4. As proposed by Honnor *et al.* (1985), digestion was performed in the presence of 200 nM adenosine to reduce day-to-day variability and to diminish cell lysis (see Hollenga *et al.*, 1989). After 1 h, cells were filtered through nylon cloth (100 μm), and washed 2–3 times with Krebs-Henseleit buffer. Adipocytes were incubated in small Teflon vessels in Krebs-Henseleit buffer at pH 7.4, containing 2% bovine serum albumin, at 37°C in a shaking waterbath gassed with 5% CO_2 in O_2. In each vessel about 50,000 cells ml^{-1} were used, as determined with a Bürker-Türk counting chamber. Before addition of the agonist, cells were preincubated for 15 min with the antagonist under investigation or with vehicle. In the case of (−)-isoprenaline, $Na_2S_2O_5$ 50 μg ml^{-1} was added to the incubation vessel as anti-oxidant. In each experiment 1, 3 and 10 μM (−)-isoprenaline was used to determine maximum lipolysis. After 90 min, the incubation was terminated by extraction with 1-propanol:*n*-heptane:1N H_2SO_4 (40:20:1). After centrifugation for 5 min at 2000 r.p.m. in a Hettich Rotixa/KS centrifuge, the upper layer was used for free fatty acid (FFA)-determination. FFA-production was measured with an auto-analyzer essentially according to Antonis (1965), as described by Hollenga *et al.* (1989). All experiments were performed in duplicate.

Data analysis

All dose-response curves were expressed relative to (−)-isoprenaline maximum.

Schild plots were constructed according to Arunlakshana & Schild (1959). When the slopes did not significantly differ from unity (two-tailed Student's *t* test, $\alpha = 0.05$), pA_2 values were calculated for each concentration of antagonist according to: $pA_2 = -\log \{[\text{antagonist}]/(DR-1)\}$ (Mackay, 1978).

ICI 118,551 concentrations were corrected for protein binding as described by Bojanic *et al.* (1985).

All data are given as mean ± s.e.mean.

Materials

Collagenase (type II, 390 u mg^{-1}) and (−)-isoprenaline hydrochloride were purchased from Sigma (St. Louis, USA). Demineralized bovine serum albumin was from Organon Technika (Oss, The Netherlands). Adenosine research grade was from Serva (Heidelberg, FRG). BRL 37344 (4-[2-[(2-hydroxy-2-(3-chlorophenyl)ethyl)amino]propyl]-phenoxyacetic acid), ICI 118,551 (erythro-1-(7-methylindan-4-yloxy)-3-(isopropylamine)-butan-2-ol), and CGP 20712A (1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-propan-2-ol) were kind gifts from Beecham (Epsom, U.K.), ICI (Macclesfield, U.K.) and Ciba-Geigy (Basel, Switzerland). All buffer salts and analytical reagents were from Merck (Amsterdam, The Netherlands).

Results

Antagonism of (−)-isoprenaline-induced lipolysis by CGP 20712A is shown in Figure 1a. With low concentrations of the antagonist, ranging from 10 nM to 10 μM, small rightward shifts of the lipolysis curves were consistently found. Only at 100 μM CGP 20712A a clear rightward shift was obtained. The Schild plot (inset) shows, as expected, a clear biphasic character. The 10 μM and 100 μM data points corresponded with a pA_2 value for CGP 20712A of 4.80 ± 0.09 ($n = 6$).

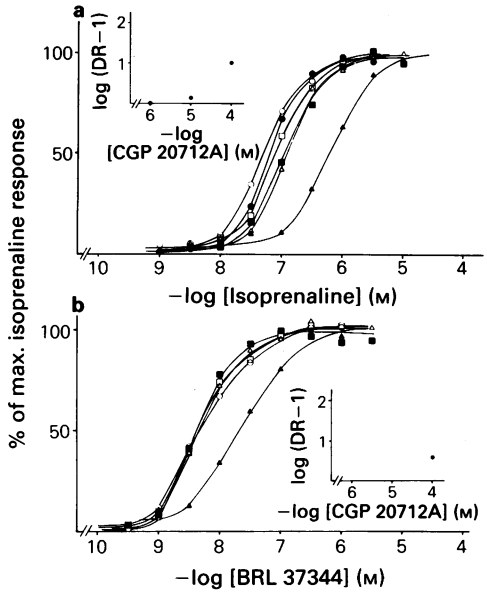

Figure 1 Antagonism of (−)-isoprenaline- (a) and BRL 37344- (b) induced lipolysis by CGP 20712A. Control (○); CGP 20712A 10 nM (●), 100 nM (□), 1 μM (■), 10 μM (△) and 100 μM (▲). Lipolysis is expressed as % relative to (−)-isoprenaline maximum. Shown are the mean of three to four experiments each performed in duplicate. For clarity standard errors are not shown, but they are less than 10%. The insets show corresponding Schild plots derived from the mean curves.

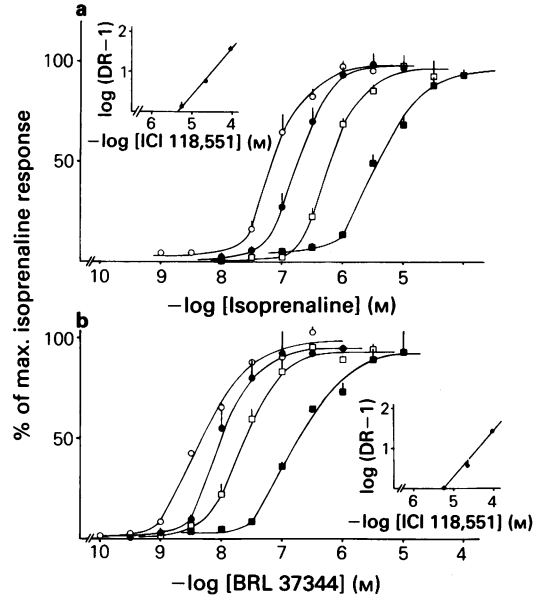

Figure 2 Antagonism of (−)-isoprenaline- (a) and BRL 37344- (b) induced lipolysis by ICI 118,551. Control (○); ICI 118,551 10 μM (●), 30 μM (□), and 100 μM (■). Lipolysis is expressed as % relative to isoprenaline maximum. Shown are the mean curves of three to four experiments each performed in duplicate. The insets show Schild plots calculated from the individual experiments. The slopes are 1.16 ± 0.07 ((−)-isoprenaline) and 1.18 ± 0.04 (BRL 37344), not significantly different from unity.

CGP 20712A did not shift BRL 37344-induced lipolysis curves, until the antagonist concentration reached 100 μM (Figure 1b). From the shift to the right induced by this concentration, a pA_2 value for CGP 20712A of 4.61 ± 0.10 ($n = 3$) was calculated.

Figure 2a shows (−)-isoprenaline-induced lipolysis antagonized by ICI 118,551. The Schild plot had a slope not significantly different from unity. The pA_2 value of ICI 118,551 was 5.49 ± 0.06 ($n = 10$).

Antagonism of BRL 37344-induced lipolysis by ICI 118,551 is shown in Figure 2b. Again, the slope of the Schild plot was not significantly different from unity, and the pA_2 value for ICI 118,551 was found to be 5.33 ± 0.06 ($n = 9$).

Discussion

Several studies with antagonists as well as with non-catecholamine agonists have cast considerable doubts on the lipolytic β-adrenoceptor being of the β_1-type, as classified by Lands *et al.* (1967) using catecholamine agonists (for a review see Zaagsma *et al.*, 1985). Thus, atypically low potencies and stereoselectivities have been found with β-adrenoceptor antagonists, irrespective of their β_1- or β_2- or non-selective properties. This was found not only in lipolysis experiments, but also with cyclic AMP-accumulation in intact cells and adenylyl cyclase in adipocyte membranes (Bojanic *et al.*, 1985). The early suggestion of a hybrid nature of the adipocyte β-adrenoceptor (Harms *et al.*, 1974) was studied in more detail by De Vente *et al.* (1980) in an elaborate study using a series of tolamolol-type antagonists. The results agreed with the hypothesis that the receptor area interacting with the aromatic moiety of β-adrenoceptor ligands is β_2 in nature while the site interacting with the alkanolamine side chain has β_1-characteristics. However, a similar approach using a series of non-catecholamine β-agonists did not confirm unequivocally the mixed β_1/β_2-character. A typical β_1-nature was not found either, even when the correlation analysis was performed with partial agonists only (i.e. the absence of receptor reserve), in agreement with previous non-catecholamine agonist studies (Zaagsma *et al.*, 1985

and references cited therein). In addition, it was established that the atypical behaviour of antagonists was not due to a heterogeneous population of both β_1- and β_2-adrenoceptors (De Vente *et al.*, 1980; Bojanic *et al.*, 1985).

In sharp contrast, radioligand displacement studies on adipocyte membranes have revealed only typical β-adrenoceptor binding sites, predominantly β_1, with high and low affinity for β_1- and β_2-selective antagonists respectively and with normal (high) stereoselectivities of enantiomer pairs (Bojanic & Nahorski, 1983). These results were recently confirmed by Bahouth & Malbon (1988) in a study in which up to four different nonselective radioligands were used. However, in all displacement experiments low radioligand concentrations, slightly above the K_d values for typical β_1- or β_2-receptors, but clearly below the atypical concentrations required to inhibit lipolysis, were used. As discussed previously (Zaagsma *et al.*, 1985), such experimental conditions will only detect the presence of typical β_1 and β_2 binding sites. In an elegant study by Bojanic & Nahorski (1984), it was indeed demonstrated that irreversible blockade of the typical β_1-receptor population was virtually without consequence on the isoprenaline-induced adenylyl cyclase activation in adipocyte membranes, implying again that the functional adipocyte response is mediated by atypical β-adrenoceptors. In addition to radioligand binding, Bahouth & Malbon (1988) also studied the inhibition of lipolysis and cyclic AMP accumulation, induced by 2 μM adrenaline, by the same two β_1- and β_2-selective antagonists, CGP 20712A and ICI 118,551 respectively, as were used in the radioligand displacement studies. The dose-inhibition curves for glycerol release appeared parallel to each other, CGP 20712A being about twice as potent as ICI 118,551. With cyclic AMP accumulation the inhibition curves were not parallel, but converged at 30 μM where both antagonists produced the same amount (50%) of inhibition. Because the onset of inhibition by CGP 20712A was at lower concentrations than with ICI 118,551 and because of the potency difference seen with glycerol release, Bahouth & Malbon (1988) concluded, on the basis of their binding and functional experiments, that the character of the rat adipocyte β-adrenoceptor is solely β_1. It was not realized, however, that atypically high (micromolar) concentrations of both antagonists were required for significant inhibition of the functional responses and also that the strong potency differences observed in the binding experiments with CGP 20712A having considerably lower K_d values for β_1-sites than ICI 118,551 were not observed at all in functional experiments.

In the present study the same antagonists were investigated for inhibition of rat adipocyte lipolysis by performing complete concentration-response curves with the agonists isoprenaline and BRL 37344. The latter compound, introduced by Arch and coworkers (1984), was reported to be 400 and 20 fold selective for rat brown adipocyte lipolysis as compared to atrial β_1- and tracheal β_2-adrenoceptor responses, respectively, the lipolytic potency being 5–6 times higher than isoprenaline. The present results show that the potency difference on rat white (epididymal) adipocytes is even higher: the control curves in Figures 1 and 2 reveal BRL 37344 to be over 10 times more potent than (−)-isoprenaline.

In these experiments CGP 20712A did not antagonize BRL 37344-induced lipolysis at concentrations up to 10 μM although at 100 μM a clear rightward shift was observed. With (−)-isoprenaline as the agonist, all CGP 20712A concentrations below 100 μM induced in a concentration-dependent manner a small but consistent shift to the right which increased dramatically at 100 μM and the resulting Schild plot was clearly biphasic. Because CGP 20712A is a very potent and highly β_1-selective antagonist, these results demonstrate (1) that BRL 37344-induced lipolysis is not mediated by typical β_1-adrenoceptors but solely by atypical β-adrenoceptors, (2) that (−)-isoprenaline-induced lipolysis is predominantly mediated by atypical receptors at all concentrations but to a small extent also by β_1-adrenoceptors and (3) that CGP 20712A up to 10 μM has no affinity for atypical adipocyte β-adrenoceptors. A comparison of the pA_2 value of CGP 20712A for rat sinoatrial β_1-adrenoceptors (9.44) as reported by Kaumann (1986), with the value for atypical rat adipocyte β-adrenoceptors found at 100 μM CGP 20172A with BRL 37344 as the agonist (4.61), reveals a 67,000 fold higher affinity of CGP 20712A for typical β_1-adrenoceptors over the atypical adipocyte β-adrenoceptors.

The experiments with ICI 118,551 (Figure 2) show that this β_2-selective antagonist also acts on adipocytes almost exclusively via atypical receptors; parallel, concentration-dependent shifts were observed, against both agonists, the slopes of the Schild plots being not significantly different from unity in both cases. The pA_2 values found (5.49 and 5.33) are well below the pA_2 values found on β_2-adrenoceptor-mediated tracheal relaxation (8.72) and on β_1-adrenoceptor-mediated atrial rate (7.19) (Arch *et al.*, 1984). Furthermore, the slightly higher (DR − 1) values observed with (−)-isoprenaline compared with BRL 37344 at all concentrations of ICI 118,551 (Figure 2, insets) are in agreement with a minor participation of β_1-adrenoceptors in the (−)-isoprenaline-induced lipolysis.

In conclusion, the remarkable difference between the affinities of CGP 20712A for typical β_1-adrenoceptors and fat cell β-adrenoceptors, found

in the present study, provides strong support for the view that rat adipocyte lipolysis is mediated predominantly by an atypical β-adrenoceptor population whereas the β_1-receptors at most play a subordinate functional role.

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References

ARCH, J.R.S., AINSWORTH, A.T., CAWTHORNE, M.A., PIERCY, V., SENNIT, M.V., THODY, V.E., WILSON, C. & WILSON, S. (1984). Atypical β-adrenoceptor on brown adipocytes as target for anti-obesity drugs. *Nature*, **309**, 163–165.

ANTONIS, A. (1965). Semiautomated method for the colorimetric determination of plasma free fatty acids. *J. Lipid Res.*, **6**, 307–312.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BAHOUTH, S.W. & MALBON, C.C. (1988). Subclassification of β-adrenergic receptors of rat fat cells: a re-evaluation. *Mol. Pharmacol.*, **34**, 318–326.

BOJANIC, D., JANSEN, J.D., NAHORSKI, S.R. & ZAAGSMA, J. (1985). Atypical characteristics of the β-adrenoceptor mediating cyclic AMP generation and lipolysis in the rat adipocyte. *Br. J. Pharmacol.*, **84**, 131–137.

BOJANIC, D. & NAHORSKI, S.R. (1983). Identification and subclassification of rat adipocyte beta-adrenoceptors using $(\pm)$-^{125}I-cyanopindolol. *Eur. J. Pharmacol.*, **93**, 235–243.

BOJANIC, D. & NAHORSKI, S.R. (1984). Irreversible antagonism of beta-adrenoceptors with para-amino-benzylcarazolol provides further evidence for an atypical rat adipocyte beta-adrenoceptor. *J. Rec. Res.*, **4**, 21–35.

DE VENTE, J., BAST, A., VAN BREE, L. & ZAAGSMA, J. (1980). β-Adrenoceptor studies. 6. Further investigations on the hybrid nature of the rat adipocyte β-adrenoceptor. *Eur. J. Pharmacol.*, **63**, 73–83.

DOOLEY, D.J., BITTIGER, H. & REYMANN, N.C. (1986). CGP 20712A: a useful tool for quantitating β_1- and β_2-adrenoceptors. *Eur. J. Pharmacol.*, **130**, 137–139.

HARMS, H.H., ZAAGSMA, J. & DE VENTE, J. (1977). Differentiation of β-adrenoceptors in right atrium, diaphragm and adipose tissue of the rat using stereoisomers of propranolol, alprenolol, nifenalol and practolol. *Life Sci.*, **21**, 123–128.

HARMS, H.H., ZAAGSMA, J. & VAN DER WAL, B. (1974). β-Adrenoceptor studies. III. On the β-adrenoceptors in rat adipose tissue. *Eur. J. Pharmacol.*, **25**, 87–91.

HOLLENGA, CH., HAAS, M., DEINUM, J.T. & ZAAGSMA, J. (1989). Discrepancies in lipolytic activities induced by β-adrenoceptor agonists in human and rat adipocytes. *Horm. Metab. Res.*, (in press).

HONNOR, R.C., DHILLON, G.S. & LONDOS, C. (1985). cAMP dependent protein kinase and lipolysis in rat adipocytes. I. Cell preparation, manipulation and predictability in behavior. *J. Biol. Chem.*, **260**, 15122–15129.

KAUMANN, A.J. (1986). The β_1-adrenoceptor antagonist CGP 20712A unmasks β_2-adrenoceptors activated by (−)-adrenaline in rat sinoatrial node. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **332**, 406–409.

LANDS, A.M., ARNOLD, A., MCAULIFF, J.P., LUDUENA, F.P. & BROWN, T.G. (1967). Differentiation of receptor systems activated by sympathomimetic amines. *Nature*, **214**, 597–598.

MACKAY, D. (1978). How should values of pA_2 and affinity constants for pharmacological competitive antagonists be estimated? *J. Pharm. Pharmacol.*, **30**, 312–313.

RODBELL, M. (1964). Metabolism of isolated fat cells. 1. Effects of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.*, **239**, 375–380.

ZAAGSMA, J., DE VENTE, J., HARMS, H.H. & JANSEN, J.-D. (1985). The nature of adipocyte beta-adrenoceptor(s). In *Pharmacology of Adrenoceptors*. ed. Szabadi, E., Bradshaw, C.M. & Nahorski, S.R., pp. 247–256. London: Macmillan Press.

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