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Relationship between lipolysis and cyclic AMP generation mediated by atypical β-adrenoceptors in rat adipocytes

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1 The nature of the β-adrenoceptor(s) mediating adenylyl cyclase activation in rat adipocyte ghosts by (−)-isoprenaline and the lipolytically selective β-adrenoceptor agonist, BRL 37344, was investigated by use of the β_1-selective antagonist, CGP 20712A. The results were compared with lipolysis in adipocytes.

2 While in lipolysis BRL 37344 was a full and 10 times more potent agonist than (−)-isoprenaline, in adenylyl cyclase activation similar pD_2 values for both agonists were found. BRL 37344 was only a partial agonist on rat adipocyte adenylyl cyclase, with an intrinsic activity of 0.62.

3 With CGP 20712A small rightward shifts of the (−)-isoprenaline concentration-response curve (CRC) were observed at concentrations up to 10 μM, while at 100 μM and 1 mM clear rightward shifts occurred. The BRL 37344 CRC was not shifted with antagonist concentrations up to 10 μM. Only at 100 μM and 1mM CGP 20712A were rightward shifts observed.

4 CGP 20712A concentrations of 10 μM and 100 μM depressed the maximum of the (−)-isoprenaline CRC to 89 and 60%, while the BRL 37344 CRCs retained the control maximum effect (62% of (−)-isoprenaline). Only at 1 mM CGP 20712A, was the CRC of BRL 37344 depressed, while the (−)-isoprenaline maximum was diminished further.

5 It was concluded that as with lipolysis, (−)-isoprenaline acts both through typical β_1- and atypical β_3-adrenoceptors for activation of adenylyl cyclase, while BRL 37344 acts solely through atypical β_3-adrenoceptors.

6 The results also demonstrate that the relationship between adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and lipolysis is different for BRL 37344 and (−)-isoprenaline. Although the maximum activation of adenylyl cyclase by BRL 37344 is only 62% of that by (−)-isoprenaline, the distance between the lipolysis and adenylyl cyclase CRCs is much larger in the case of BRL 37344, indicating a larger transduction reserve for this agonist.

Introduction

It is now well established that rat adipocyte lipolysis is predominantly mediated by atypical β-adrenoceptors (Zaagsma & Nahorski, 1990, and references cited therein). An atypical or hybrid β-adrenoceptor was already suggested by Harms *et al.* (1974) and by De Vente *et al.* (1980), who demonstrated low affinity of this adrenoceptor for both β_1- and β_2-selective and non-selective antagonists. In addition, for the adipocyte β-adrenoceptor atypically low stereoselectivity ratios of antagonist enantiomers were found compared to typical β_1- (cardiac) and β_2- (skeletal muscle) adrenoceptors (Harms *et al.*, 1977). The introduction of a novel series of β-adrenoceptor agonists that were lipolytically selective (Arch *et al.*, 1984) strengthened the hypothesis of an atypical β-adrenoceptor mediating lipolysis. Important progress in this field was made by the recent isolation of a human gene coding for a β_3-adrenoceptor with clear atypical β-adrenoceptor properties when expressed in chinese hamster ovary cells (Emorine *et al.*, 1989).

Recently, direct evidence was obtained that rat adipocyte lipolysis is indeed mediated predominantly by atypical β-adrenoceptors, while the typical β_1-adrenoceptors play at most a small, subordinate role (Hollenga & Zaagsma, 1989). It was found that when (−)-isoprenaline-induced lipolysis was antagonized with the highly β_1-selective antagonist, CGP 20712A, small but consistent shifts to the right of the concentration-response curves (CRCs) occurred at antagonist concentrations up to 10 μM, while only at 100 μM CGP 20712A was a clear rightward shift found. In contrast, with the lipolytically selective compound, BRL 37344, which was shown to be about 10 times more potent in lipolysis than (−)-isoprenaline, no rightward shifts of the CRC were observed at CGP 20712A concentrations up to 10 μM. Only at 100 μM CGP 20712A was a clear rightward shift found. It was concluded that the main action of (−)-isoprenaline is through atypical β-adrenoceptors, and a small part through typical β_1-adrenoceptors, while BRL 37344 acts solely through atypical β-adrenoceptors (Hollenga & Zaagsma, 1989).

In the present study the activation of adenylyl cyclase in rat adipocyte ghosts by (−)-isoprenaline and BRL 37344 was investigated. Both agonists were also studied in the presence of increasing concentrations of CGP 20712A, and interesting differences between lipolysis and adenylyl cyclase activation were observed.

Methods

Adipocyte ghosts isolation

Epididymal fat pads from male Wistar rats (210–230 g) were used. Adipocytes were isolated as described (Hollenga & Zaagsma, 1989), except that no adenosine was used in the isolation medium. Immediately after fat cell isolation, adipocyte ghosts were prepared by lysis essentially as described by Bojanic *et al.* (1985). The isolated adipocytes were lysed in 20 volumes of Tris-HCl 2 mM, EGTA 2 mM, pH 7.4, at room temperature for 15 min, followed by centrifugation at 50,000*g* for 20 min at 4°C. Supernatant and congealed fat cake were removed and the pellet was washed with ice-cold Tris-HCl 80 mM, $MgSO_4$ 4 mM, EDTA 0.2 mM, isobutyl methylxanthine (IBMX) 0.4 mM, pH 7.4, and centrifuged again at 50,000*g* for 20 min at 4°C. The final pellet was resuspended in the same buffer solution and stored in liquid nitrogen. Protein concentration was determined according to Lowry *et al.* (1951), with bovine serum albumin used as standard.

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Adenylyl cyclase stimulation

Adenylyl cyclase stimulation experiments were carried out by incubating 20–50 μg protein for 20 min at 37°C in a buffer solution composed of Tris-HCl 120 mM, $MgCl_2$ 3.5 mM, adenosine 5'-triphosphate (ATP) 1 mM, dithiotreitol 1 mM, phosphocreatinine 20 mM, creatine phosphokinase 5 u, guanosine 5'-triphosphate (GTP) 0.1 mM and IBMX 0.5 mM, with the appropriate concentrations of drugs under investigation, in a final volume of 100 μl. When (−)-isoprenaline was used as agonist, $Na_2S_2O_5$ 50 $\mu g\,ml^{-1}$ was added as antioxidant. The incubation was terminated by adding 400 μl Tris-HCl 50 mM, EDTA 4 mM, pH 7.4, and subsequently placing the test tubes in a boiling waterbath. After 10 min the tubes were placed on ice and were centrifuged for 5 min at 4°C at 2000g. The supernatant was stored in polyethylene vials at −20°C until the adenosine 3':5'-cyclic monophosphate (cyclic AMP) determination was carried out.

Isolation of cyclic AMP binding protein

Fresh bovine adrenals, obtained from the local slaughterhouse, were transported to the laboratory on ice. Fat and medulla were removed and the adrenal cortices were homogenized with an Ultra-Turrax, in 2 volumes of Littlefields medium, composed of sucrose 250 mM, Tris 50 mM, KCl 25 mM, $MgCl_2$ 5 mM, 2-mercapto-ethanol 6 mM and theophylline 8 mM, pH 7.4. The homogenate was centrifuged for 5 min at 2000g at 4°C. The supernatant was centrifuged at 50,000g for 20 min at 4°C. The supernatant remaining after this centrifugation step was lyophilized and stored at −20°C.

Determination of cyclic AMP

Determination of cyclic AMP production was carried out essentially according to Brown *et al.* (1971) by incubating the samples with 1.7 mg bovine adrenal binding protein and 10 μCi [^{3}H]-cyclic AMP in a buffer composed of Tris-HCl 50 mM, EDTA 4 mM, pH 7.4 in a final volume of 300 μl for at least 3 h at 4°C to reach equilibrium. When necessary, samples were diluted with incubation buffer. The incubation was terminated by mixing with charcoal suspension (Norit A special; $2.5\,g\,l^{-1}$) at 4°C, mixing, and subsequent centrifugation to remove unbound [^{3}H]-cyclic AMP. The upper layer was put in scintillation vials and 3 ml scintillation fluid was added. The samples were counted for 5 min in a Beckman type LS 1701 liquid scintillation counter with an efficiency of 46%.

Data analysis

Data are presented as means ± s.e.mean. Dose-response curves of cyclic AMP production are 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, $P < 0.05$), pA_2 values were calculated for each concentration of antagonist according to: $pA_2 = -\log\{[\text{antagonist}]/(DR - 1)\}$ (MacKay, 1978).

Materials

Collagenase (type II, $390\,u\,mg^{-1}$), (−)-isoprenaline hydrochloride, dithiotreitol, phosphocreatine, creatinephophokinase, GTP, IBMX, 2-mercapto-ethanol, theophylline and cyclic AMP were purchased from Sigma (St. Louis, U.S.A.). Demineralized bovine serum albumin was from Organon Technika (Oss, The Netherlands). All buffer salts were from Merck. [^{3}H]-cyclic AMP was from The Radiochemical Center (Amersham, UK). Scintillation fluid (Picofluor) was purchased from Packard Instruments B.V. (Groningen, The Netherlands).

BRL 37344 (4-[2-[(2-hydroxy-2-(3-chlorophenyl)ethyl)-amino]propyl]-phenoxyacetic acid) 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 of Beecham (Epsom, U.K.) and Ciba Geigy (Basle, Switzerland), respectively.

Results

Adenylyl cyclase stimulation of rat white adipocyte ghosts by (−)-isoprenaline and BRL 37344 was linear with time and protein concentration (data not shown). Basal cyclic AMP production was 0.66 ± 0.17, maximal cyclic AMP production by (−)-isoprenaline was $2.89 \pm 0.69\,nmol\,mg^{-1}$ protein $20\,min^{-1}$ ($n = 6$). In Figure 1 the concentration-response curves (CRCs) for both agonists are depicted together with the lipolytic CRCs in rat isolated adipocytes, taken from our previous study (Hollenga & Zaagsma, 1989). In lipolysis, both compounds were full agonists, with pD_2 values ($-\log EC_{50}$) of 7.28 ± 0.11 for (−)-isoprenaline and 8.34 ± 0.07 for BRL 37344, the latter being 10 times more potent. Surprisingly, the pD_2 values for adenylyl cyclase activation were similar for both agonists, being 6.07 ± 0.10 for (−)-isoprenaline and 5.99 ± 0.24 for BRL 37344. Moreover, compared to (−)-isoprenaline, BRL 37344 was a partial agonist on adenylyl cyclase stimulation, with an intrinsic activity of 0.62.

When the CRCs were performed with increasing concentrations of CGP 20712A, differences between (−)-isoprenaline and BRL 37344 were found. From 100 nM to 10 μM CGP 20712A small rightward shifts of the (−)-isoprenaline CRC were observed. At higher antagonist concentrations of 100 μM and 1 mM the rightward shifts enlarged. Furthermore, with these CGP 20712A concentrations a depression of the maximum response of the (−)-isoprenaline CRC was found. The Schild plot constructed from these data was clearly biphasic, the slope of the steep part being 0.80 ± 0.15, not significantly different from unity. From the shifts with 100 μM and 1 mM antagonist, after subtraction of the 'high affinity part' of the Schild plot (Bond & Clarke, 1988), a pA_2 value for CGP 20712A for antagonizing (−)-isoprenaline-induced cyclic AMP production by the higher concentrations of the antagonist of 4.53 ± 0.17 ($n = 8$) was calculated (Figure 2a).

With BRL 37344 different results were found. No significant shift of the BRL 37344 CRC was observed up to 10 μM CGP 20712A. At 100 μM antagonist a small rightward shift was found, which increased at 1 mM CGP 20712A. With BRL 37344, no depression of the CRC occurred up to 100 μM of the antagonist. Only at 1 mM CGP 20712A was a clear depression of the CRC seen. The Schild plot constructed from the data obtained with 100 μM and 1 mM CGP 20712A had a slope of

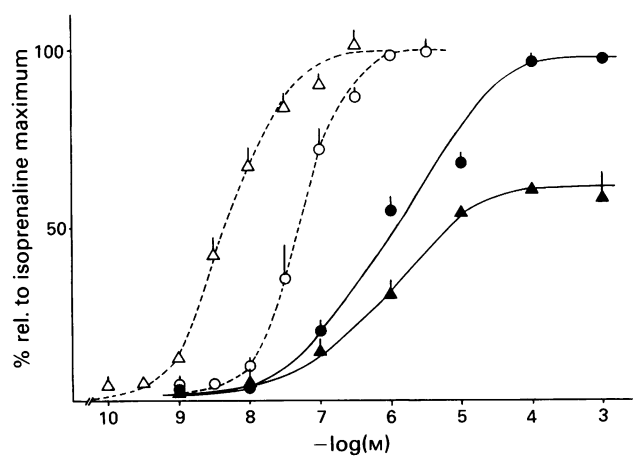

Figure 1 Lipolysis (open symbols) and adenosine 3':5'-cyclic monophosphate (cyclic AMP) production (closed symbols) in Wistar rat adipocytes and adipocyte ghosts induced by (−)-isoprenaline (○, ●) and BRL 37344 (△, ▲). Both lipolysis and cyclic AMP production are expressed as % relative to (−)-isoprenaline maximum.

1.20 ± 0.19, which was also not significantly different from unity. A pA_2 value of 4.60 ± 0.17 ($n = 6$) was calculated for CGP 20712A antagonizing BRL 37344 induced adenylyl cyclase stimulation (Figure 2b).

Discussion

The present study provides information on two steps involved in β-adrenoceptor-mediated lipolysis in rat adipocytes. First, the activation of adenylyl cyclase by (−)-isoprenaline and BRL 37344, and second, the relationship between cyclic AMP generation and lipolysis for both agonists.

One of the most striking observations is that BRL 37344, while being a full and 10 fold more potent agonist than (−)-isoprenaline on lipolysis, is only a partial agonist on cyclic AMP production, with a similar potency to (−)-isoprenaline. For BRL 37344 a 220 fold difference in ED_{50} values for lipolysis and cyclic AMP generation was found, while for (−)-isoprenaline only a 16 fold difference was obtained. As a consequence, maximum lipolysis induced by BRL 37344 is already reached at 20% of the maximum cyclic AMP production, whereas with (−)-isoprenaline maximum lipolysis required 50%.

When adenylyl cyclase stimulation by (−)-isoprenaline and BRL 37344 was antagonized with the β_1-selective antagonist CGP 20712A, a similar pattern was observed to that seen with lipolysis (Hollenga & Zaagsma, 1989). With low antagonist concentrations of 100 nM to 10 μM, small rightward shifts of the (−)-isoprenaline CRC were found, indicating a small contribution of typical β_1-adrenoceptors. The rightward shift increased when higher antagonist concentrations of 100 μM to 1 mM were applied. While with BRL 37344 no significant shift was observed at concentrations up to 10 μM, at 100 μM and 1 mM concentrations of CGP 20712A rightward shifts were found. The pA_2 values calculated for antagonism of cyclic AMP production by the higher CGP 20712A concentrations were 4.53 and 4.60, being similar to the pA_2 values of 4.80 and 4.61 reported for CGP 20712A antagonism of (−)-isoprenaline- and BRL 37344-induced lipolysis, respectively (Hollenga & Zaagsma, 1989). Because of the similarity of the findings in the present study on cyclic AMP generation with those reported for lipolysis (Hollenga & Zaagsma, 1989) it can be concluded that adenylyl cyclase activation is also predominantly mediated by atypical β-adrenoceptors, with a small contribution of the typical β_1-adrenoceptors. BRL 37344 acts solely through the atypical β-adrenoceptors.

In the presence of 10 μM CGP 20712A, i.e. under complete β_1-adrenoceptor blockade, the CRCs for lipolysis (Hollenga & Zaagsma, 1989) and adenylyl cyclase activation (Figure 2) revealed that with (−)-isoprenaline the difference in EC_{50} values increased from 16 fold to 50 fold, whereas with BRL 37344 a 295 fold difference was found, comparable to the value found in the absence of CGP 20712A. Thus, the marked discrepancy between cyclic AMP generation and lipolysis observed with both agonists, but in particular with BRL 37344, was entirely due to occupation of the atypical β-adrenoceptors.

This discrepancy could be explained by functional compartmentalization of cyclic AMP, which implies more efficient activation of protein kinase A involved in triglyceride lipase phosphorylation in the case of BRL 37344 (Honnor *et al.*, 1985). The existence of functional and non-functional cyclic AMP compartments in fat cells was already indicated by the results of Schimmel (1984). With hamster adipocytes this author found that 1 μM forskolin, a diterpene acting directly on adenylyl cyclase, while producing relatively large amounts of cyclic AMP, hardly stimulated lipolysis. When 0.01 μM isoprenaline was added, which itself did not stimulate cyclic AMP production, the two compounds acted synergistically on lipolysis but not on cyclic AMP generation, and glycerol production dramatically increased. It was concluded that this potentiation could arise if isoprenaline directed the cyclic AMP produced in response to forskolin into the appropriate compartment coupled to lipolysis. Alternatively, the coexistence of cyclic AMP-dependent and cyclic AMP non-dependent mechanisms might be responsible for the observed differences with BRL 37344 and (−)-isoprenaline, BRL 37344 being the most potent in stimulating the cyclic AMP non-dependent mechanism. On the basis of the differential inhibition by the adenosine A_1-receptor agonist N^6-phenylisopropyladenosine of lipolysis and adenylyl cyclase activation induced by isoprenaline as compared to forskolin, Allen & Quesenberry (1988) also considered the possibility of a cyclic AMP-independent mechanism. In addition, a second regulatory factor, acting synergistically with cyclic AMP, might by involved, BRL 37344 again being more potent in activating this factor than (−)-isoprenaline.

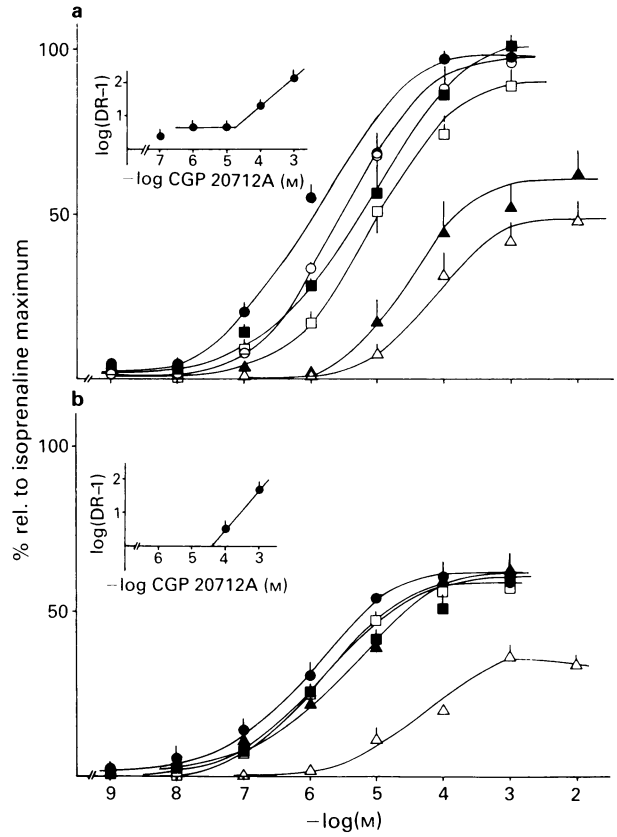

Figure 2 Antagonism of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) production in Wistar rat adipocyte ghosts, induced by (−)-isoprenaline (a) and BRL 37344 (b), with CGP 20712A. Control (●), CGP 20712A 100 nM (○), 1 μM (■), 10 μM (□), 100 μM (▲), and 1 mM (△). Cyclic AMP production is expressed as % relative to (−)-isoprenaline maximum. The insets show Schild plots calculated from the individual experiments.

Interestingly, in rat brown fat tissue even larger differences between adenylyl cyclase activation and stimulation of the respiratory rate were observed (Muzzin *et al.*, 1988). From the EC_{50} values reported in that paper, for BRL 37344, being again a partial agonist on adenylyl cyclase, a difference of 5111 fold between cyclic AMP generation and increase in oxygen consumption could be calculated and for (−)-isoprenaline a 1521 fold difference, indicating a tighter coupling of the functional cyclic AMP pool and/or alternative factors with the respiration stimulation of brown adipocytes in the case of BRL 37344.

It is known that competitive antagonists at high concentrations can act non-competitively, resulting in depression of the CRCs, particularly when applied with agonists having a low intrinsic activity (Kenakin, 1984). Bojanic & Nahorski (1984) observed a dose-dependent depression of rat reticulocyte adenylyl cyclase activation by isoprenaline with the photo-

affinity β-adrenoceptor antagonist *para*-aminobenzylcarazolol (*p*ABC). They noticed however, that there was no difference in *p*ABC behaviour with or without N-succinimidyl-6-(4'-azido-2'-nitrophenylamino)-hexanoate (SANAH) and photolysis, indicating that the depression of maximal adenylyl cyclase activity occurred during (slowly?) reversible interaction of the antagonist with the β-adrenoceptor, instead of being a result of irreversible β-adrenoceptor blockade.

In the present study, CGP 20712A at high concentrations caused depression of the CRCs of both agonists. However, (−)-isoprenaline-induced maximum cyclic AMP production already started to decrease at 10 μM CGP 20712A, while the BRL 37344 CRC was found to be depressed only at 1 mM CGP 20712A. Since (−)-isoprenaline activates both the atypical and the typical β_1-adrenoceptor, the total amount of cyclic AMP produced after stimulation with this agonist could be the sum of the production by the individual β-adrenoceptors. The atypical β-adrenoceptor, maximally activated by BRL 37344, contributes 62% to the maximum amount obtained with (−)-isoprenaline. At 100 μM CGP 20712A the β_1-adrenoceptor-mediated contribution by (−)-isoprenaline is totally depressed, while the activation of the atypical β-adrenoceptor, which is known to have a low affinity for the antagonist (Hollenga & Zaagsma, 1989), is still maximal, as can be seen from the experiments with BRL 37344 in the absence and presence of CGP 20712A of 100 μM and lower. The observation that at 100 μM CGP 20712A, (−)-isoprenaline still stimulates adenylyl cyclase to 60% of its own maximum response, comparable to the control maximum BRL 37344 activity, whereas at 1 mM both the (−)-isoprenaline and the BRL 37344 response are depressed, supports this view.

In conclusion, the present study shows that, as already demonstrated for lipolysis, adenylyl cyclase activation in rat adipocytes by BRL 37344 is solely and by (−)-isoprenaline is predominantly mediated by atypical β_3-type adrenoceptors. Although this adrenoceptor apparently is not very efficiently coupled to adenylyl cyclase, given the maximum activity of 62% compared to (−)-isoprenaline, the generated cyclic AMP is activating the triglyceride lipase-sensitive pool of protein kinase A very well. In the case of BRL 37344, this results in a larger distance between the lipolysis and adenylyl cyclase CRCs than in the case of (−)-isoprenaline, implying that the high lipolytic activity of BRL 37344 is due to a larger transduction reserve. It would be most interesting to investigate this relationship in other cell types.

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