Differences in the properties of A₁-type adenosine receptors in rat white and brown adipocytes

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1. White adipocytes were found to be more responsive than brown adipocytes to inhibition of lipolysis by the A₁ adenosine receptor agonist phenylisopropyladenosine. 2. Radioligand binding studies with plasma membranes isolated from the two adipocyte types indicated differences in the properties of the A₁ receptors. *K_d* values (high and low affinity) for phenylisopropyladenosine were higher in membranes from brown adipocytes. The *K_d* values for the antagonist dipropylcyclopentylxanthine were also higher in brown adipocyte membranes. 3. The effects of guanine nucleotides in converting adipocyte A₁ receptors into a low-affinity state were enhanced by dithiothreitol.

INTRODUCTION

Adenosine acts as a paracrine agent to inhibit lipolysis in white adipose tissue cells. This effect is mediated through an A₁ type of adenosine receptor coupled to the guanine-nucleotide-binding protein (G-protein) G_i (Londos *et al.*, 1980; Murayama & Ui, 1983; Olansky *et al.*, 1983; Moreno *et al.*, 1983). Binding of A₁-receptor-specific agonists to white adipocyte plasma membranes has been demonstrated (Trost & Schwabe, 1981; Chohan *et al.*, 1984; Malbon *et al.*, 1985).

In brown adipose tissue, activation of lipolysis by noradrenaline released from sympathetic nerve terminals is regarded as being an essential early event in the stimulation of thermogenesis (Nicholls & Locke, 1983, 1984; Cannon & Nedergaard, 1985). In this tissue also, adenosine has been shown to inhibit lipolysis, thereby diminishing the stimulation of brown adipocyte respiration by noradrenaline (Szillat & Buckowiecki, 1983; Schimmel & McCarthy, 1984; Woodward & Saggerson, 1986; Schimmel *et al.*, 1987; Jamal & Saggerson, 1988; Jamal *et al.*, 1988).

In earlier studies from this laboratory, it appeared likely that the A₁ adenosine receptor agonist *N*⁶-L-phenylisopropyladenosine (PIA) was a more effective inhibitor of lipolysis in white adipocytes than in brown fat cells. In the present study we have demonstrated this directly. Following this we have investigated the binding of the selective A₁ receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) and of the agonist PIA to plasma membranes isolated from both white and brown adipocytes. Differences in the characteristics of binding between the two cell types are demonstrated.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained and treated as described by Honnor & Saggerson (1980) and Woodward & Saggerson (1986). In addition, PIA, NECA [5'-(*N*-ethyl)carboxamidoadenosine], GTP and Gpp[NH]p (guanosine 5' [β -imidotriphosphate]) were from the Boehringer Corporation (London) Ltd. (Lewes, East Sussex, U.K.), and [³H]PIA and [³H]DPCPX were from Amersham International (Little Chalfont, Bucks., U.K.).

Animals

The animals used were 6-week-old male Sprague–Dawley rats (160–180 g) bred at University College London. All animals had constant access to drinking water and to Rat & Mouse No. 3 Breeding Diet (Special Diet Services, Witham, Essex, U.K.) which contained (by wt.) 21% digestible crude protein, 4% digestible crude oil and 39% starches and sugars. The light/dark cycle was 13 h/11 h, with light from 06:00 to 19:00 h.

Isolation of adipocytes

White adipocytes were isolated from epididymal adipose tissue essentially as described by Rodbell (1964). Brown adipocytes were isolated from the interscapular depot of the same animals (Woodward & Saggerson, 1986) by the procedure originally described by Fain *et al.* (1967) and elaborated by Nedergaard & Lindberg (1982).

Preparation of adipocyte plasma membranes

Brown or white adipocytes isolated from several rats were broken in ice-cold 0.25 M-sucrose/10 mM-Tris/HCl/2 mM-EGTA (pH 7.4) using a Potter–Elvehjem glass homogenizer fitted with a Teflon pestle (6 up-and-down strokes at 500 rev./min and radial clearance of 0.2 mm). The resulting homogenates were fractionated by the method of Belsham *et al.* (1980), which employs density-gradient centrifugation in Percoll. Finally, the membrane preparations were suspended in 50 mM-Tris/HCl buffer (pH 7.4) containing 1 mM-MgCl₂ ('Tris/Mg buffer') and stored at –70 °C until required. The protein contents of these preparations were measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. The degree of purity of white adipocyte plasma membrane preparations has been discussed previously (Chohan & Saggerson, 1982). When 30 000 g × 30 min crude particulate fractions obtained from homogenates of brown adipocytes were fractionated using Percoll, 90% of the 5'-nucleotidase activity was recovered in the plasma membrane fraction. However, this fraction also contained 25% of the applied succinate-cytochrome *c* reductase activity. The brown adipocyte plasma membrane preparation was therefore appreciably contaminated with membranes derived from mitochondria.

Abbreviations used; *B_{max}*, maximum binding capacity at a given site; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; DPX, 1,3-diethyl-8-phenylxanthine; Gpp[NH]p, guanosine 5' [β -imidotriphosphate]; G-protein, guanine-nucleotide-binding protein; NECA, 5'-(*N*-ethyl)-carboxamidoadenosine; PIA, *N*⁶-L-phenylisopropyladenosine; XAC, 1,3-dipropyl-8-[4-[(aminoethyl)aminocarbonyl]methoxy]-phenyl]-xanthine.

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Measurement of lipolysis

Portions of the brown or white adipocyte preparations were incubated with shaking at 37 °C in 25 ml silicone-treated flasks containing 4 ml of Krebs–Henseleit (1932) saline, 4% (w/v) fatty-acid-poor albumin, 5 mM-glucose and adenosine deaminase (1 unit/ml). Other additions are indicated in the legend to Fig. 1. The gas phase was O₂/CO₂ (19: 1). After 60 min the flask contents received HClO₄ to a final concentration of 6% (w/v) and were neutralized and treated as described by Fernandez & Saggerson (1978). Neutralized extracts were assayed for glycerol by the method of Garland & Randle (1962).

Measurement of radioligand binding to plasma membranes

The procedure was essentially as described by Chohan *et al.* (1984). Plasma membranes in Tris/Mg buffer (0.2 mg of protein/ml) were preincubated with adenosine deaminase (3 units/ml) for 10 min at 30 °C before being used immediately. The binding assays contained 75 µg of plasma membrane protein incubated at 37 °C for 15 min in 0.4 ml of Tris/Mg buffer containing [³H]DPCPX or [³H]PIA and other additions as indicated in individual Figures. After incubation, the membranes were separated from the medium and washed using a programmable Brandel cell harvester. Filtration under vacuum was through Whatman GF/B glass-fibre filters which were then washed with 2 × 5 ml of Tris/Mg buffer before being taken for scintillation counting.

Statistical methods

Values shown in Figures are means of the indicated number (*n*) of separate cell or membrane preparations. The bars in Figures indicate S.E.M. values; where these are not shown they lie within the symbol.

Saturation and competition data for radioligand binding were analysed by a non-linear weighted least squares curve-fitting procedure using a search-type minimization method (Colquhoun, 1971). On input of data as means ± S.E.M., the programme provided estimates of *K_d* and *B_{max}*, and, in the case of competition curves, non-specific binding. Statistical analysis to assess the appropriateness of one- or two-site models of binding was performed using a partial *F* test (Munson & Rodbard, 1980).

RESULTS AND DISCUSSION

In the presence of a high concentration of adenosine deaminase (1 unit/ml) to remove endogenous adenosine, brown and white adipocytes isolated from the same animals were exposed to sufficient noradrenaline to activate lipolysis to 90% of its maximum rate. As commented on previously (Jamal & Saggerson, 1988) white adipocyte lipolysis is more responsive to low concentrations of noradrenaline, so that 30 nM and 110 nM concentrations of the neurotransmitter were needed to achieve 90% stimulation in the white and brown cells respectively. Fig. 1 shows the dose-response curves for inhibition of lipolysis by the A₁ adenosine receptor agonist PIA. In the case of white adipocytes, 50% inhibition was achieved with 2.5 nM-PIA, and higher concentrations (0.1–1 µM-PIA) caused up to 95% inhibition of the process. By contrast, a significantly higher concentration of PIA (7.5 nM) was required to inhibit brown adipocyte lipolysis by 50%, and maximally effective concentrations of the agonist (1–10 µM-PIA) only inhibited the process by 80%.

DPCPX has been shown to be an adenosine receptor antagonist with high selectivity for the A₁ rather than the A₂ type of receptor (Lee & Reddington, 1986; Bruns *et al.*, 1987). As shown in Fig. 2, this compound bound to both brown and white adipocyte plasma membranes with the characteristics of a single class of site

Fig. 1 Inhibition of lipolysis in brown and white adipocytes by PIA

All incubations contained noradrenaline at concentrations sufficient to cause stimulation of lipolysis to 90% of maximal (110 nM and 30 nM for brown and white adipocytes respectively). ○, White adipocytes (*n* = 5). IC₅₀ = 2.5 ± 0.5 nM. ●, Brown adipocytes (*n* = 5). IC₅₀ = 7.5 ± 0.6 nM (*P* < 0.001 versus the value for white adipocytes).

Fig. 2. Specific binding of [³H]DPCPX to plasma membranes from brown and white adipocytes

Non-specific binding was determined in the presence of 0.1 nM unlabelled PIA and was subtracted from the measured total binding to give the values shown. Scatchard plots were linear (*r* = −0.96 and *r* = −0.93 for brown and white adipocyte membranes respectively) and gave the following values for binding parameters. White adipocytes (○, *n* = 5): *K_d* = 1.4 ± 0.2 nM, *B_{max}* = 996 ± 89 fmol/mg of protein; brown adipocytes (●, *n* = 5): *K_d* = 8.4 ± 0.7 nM, *B_{max}* = 543 ± 52 fmol/mg of protein (*P* < 0.01 versus the value for white adipocytes).

in each case. Relative to membrane protein, the abundance of DPCPX binding sites was approx. 50% lower in the membranes from the brown fat cells. This difference must, in part at least, be explained by the somewhat greater contamination of the preparation by other membranes (see above). It is not possible from the present data to establish whether the brown fat cell membranes have an intrinsically lower density of the A₁ receptor. Surprisingly, the *K_d* for DPCPX was 6-fold (*P* < 0.001) higher in the brown adipocyte membranes. This large difference is unlikely to be attributable to the degree of contamination of the fraction

Fig. 3. Competition for DPCPX binding sites on adipocyte plasma membranes by PIA or NECA

All binding assays contained 2 nM-[³H]DPCPX. The fitted displacement curves are computer estimates. Values of *n* and estimates of *K_d* are shown in Table 1. (a) White adipocyte membranes, (b) brown adipocyte membranes. The displacing ligands are indicated by: ○, PIA; ●, PIA in the presence of 10 μM-GTP and 1 mM-dithiothreitol; ■, NECA.

Table 1. Competition for DPCPX binding sites on adipocyte plasma membranes by PIA or NECA

The values relate to the experiments shown in Figs. 3(a) and 3(b).

Adipocyte type	<i>n</i>	Displacing ligand	GTP	Preferred displacement model	<i>K_d</i> values for displacing ligand (nM)		Receptors in high-affinity state (% of total)
					High affinity	Low affinity	
White	6	PIA	—	2 site (<i>P</i> < 0.05)	0.16 ± 0.12	12.3 ± 4.9	60
	5	PIA	+	1 site	—	9.6 ± 2.2	0
	5	NECA	—	1 site	—	10.8 ± 1.7	0
Brown	5	PIA	—	2 site (<i>P</i> < 0.025)	3.2 ± 0.9	422 ± 160	83
	5	PIA	+	1 site	—	283 ± 108	0
	5	NECA	—	1 site	—	8.1 ± 1.8	0

that was observed, and must therefore represent a difference in the intrinsic properties of the receptor between the two cell types. PIA displaced 2 nM-[³H]DPCPX from both types of plasma membrane (Figs. 3a and 3b). Approx. 10–15% of the DPCPX binding was accounted for by non-specific binding in white adipocyte membranes (Fig. 3a), whereas this value was higher (approx. 40–50%) in membranes from brown fat cells (Fig. 3b). With PIA as the competing ligand in the absence of GTP a two-site model of displacement was found to be more suitable using the *F* test (Table 1). In the case of membranes from white adipocytes, 60% of the receptors were in a high-affinity state, and the *K_d* values for PIA (calculated using a *K_d* value for DPCPX of 1.4 nM) were: *K_{d1}* = 0.16 nM and *K_{d2}* = 12.3 nM. That these are reasonable estimates was shown independently by direct measurement of the saturation binding of [³H]PIA to white adipocyte membranes (Fig. 4). In this case 47% of the receptors were in a high-affinity state, and the directly measured values for the binding parameters (*K_{d1}* = 0.45 nM and *K_{d2}* = 25 nM) were in reasonable agreement with those calculated from the competition study (Fig. 3a). It is noteworthy that Ramkumar & Stiles (1988) have recently calculated values of *K_{d1}* = 1 nM and *K_{d2}* = 60 nM, with 81% of the receptors in the high-affinity state, for displacement of the antagonist [³H]XAC by PIA in rat white adipocyte membranes. When studying displacement of the non-selective (*A₁* and *A₂*) adenosine receptor antagonist [³H]DPX (1,3-diethyl-8-phenylxanthine) by PIA in rat brain membranes,

Lohse *et al.* (1984) observed that GTP converts the receptor population into the low-affinity state. In addition, Ramkumar & Stiles (1988) observed a rightwards shift in the curve for displacement of [³H]XAC by PIA when 100 μM-Gpp[NH]p was added to white adipocyte membranes. In preliminary experiments we were unable to observe any shift in the displacement of [³H]DPCPX by PIA when GTP was added to white or brown adipocyte membranes. This lack of effect could not be attributed to GTPase activity in the membranes, because the non-hydrolysable analogue Gpp[NH]p was also ineffective. However, 10 μM-GTP was found to cause pronounced rightwards shifts in the displacement curves when 1 mM-dithiothreitol was present (Figs. 3a and 3b). Dithiothreitol alone did not have any effect on the displacement curves in the absence of GTP, nor did it alter the binding of DPCPX (results not shown). Table 1 shows that a single-site displacement model was preferred by the *F* test when GTP (with dithiothreitol) was present and, for white adipocyte membranes, the calculated *K_d* value for PIA of 9.6 nM was similar to the low-affinity *K_d* value in the absence of GTP. In the case of membranes from brown adipocytes, again a two-site displacement model was preferred with PIA alone, with a single-site model in the presence of GTP (and dithiothreitol). However, the high-affinity and low-affinity *K_d* values for PIA (calculated using a *K_d* value for DPCPX of 8.4 nM) were 20–36-fold larger than those found with membranes from white adipocytes. As shown in Figs. 4 and 5, we also examined directly the binding of [³H]PIA

Fig. 4. Specific binding of [3 H]PIA to plasma membranes from white and brown adipocytes

Non-specific binding was determined in the presence of 0.1 μ M unlabelled PIA and was subtracted from the measured total binding to give the values shown. The binding curves are computer estimates. $\circ$, White adipocyte membranes ($n = 6$). Using the F test, a two-site model was favoured ($P < 0.005$). The estimated binding parameters were: $K_{d1} = 0.45 \pm 0.06$ nM, $B_{max,1} = 584 \pm 30$ fmol/mg of protein; $K_{d2} = 25 \pm 7$ nM, $B_{max,2} = 646 \pm 50$ fmol/mg of protein. The inset is a Scatchard plot of these data. $\bullet$, Brown adipocyte membranes ($n = 3$). The computer analysis indicated a high-affinity site of $K_d = 2.3 \pm 0.5$ nM and $B_{max} = 86 \pm 6$ fmol/mg of protein. Other site(s) were not readily saturable. These data are also represented as a Scatchard plot in Fig. 5.

Fig. 5. Scatchard plot of [3 H]PIA binding to brown adipocyte plasma membranes

For details, see the legend to Fig. 4.

to brown adipocyte membranes. A high-affinity site with $K_d = 2.3$ nM and $B_{max} = 86$ fmol/mg of protein was indicated by the computer analysis. This K_d value was in quite good agreement with the value of 3.2 nM deduced from the displacement analysis (Table 1). The remainder of the PIA binding to these membranes was at a very-low-affinity site(s) ($K_d > 1$ μ M) that could not be saturated. The experimental reason for this failure to saturate was that, with 1 μ M [3 H]PIA, non-specific binding exceeded 80 %

Fig. 6. Effect of Gpp[NH]p in decreasing PIA binding to white adipocyte plasma membranes

Specific binding was measured at the indicated concentrations of PIA in the presence and absence of 100 μ M-Gpp[NH]p ($n = 4$): $\bullet$, with 1 mM-dithiothreitol; $\circ$, without dithiothreitol. Absolute values for the decreases in PIA binding (fmol/mg of protein) at the following concentrations of PIA were: with dithiothreitol: 0.1 nM, 75 ± 9 ; 0.3 nM, 144 ± 17 ; 1 nM, 243 ± 21 ; 3 nM, 526 ± 57 ; 10 nM, 703 ± 64 ; 30 nM, 861 ± 73 ; 100 nM, 640 ± 82 ; without dithiothreitol: 0.1 nM, 105 ± 16 ; 0.3 nM, 230 ± 28 ; 1 nM, 330 ± 37 ; 3 nM, 630 ± 42 ; 10 nM, 650 ± 51 ; 30 nM, 260 ± 37 ; 100 nM, 120 ± 31 .

of the total binding and it therefore was not feasible to test with higher concentrations of PIA.

Garcia-Sainz & Torner (1985) have reported that white adipocytes possess both A_1 and A_2 types of adenosine receptors. Fig. 3 and Table 1 show that the A_2 -receptor-selective ligand NECA displaced [3 H]DPCPX from brown and white adipocyte membranes. However, the displacement curves were distinctly of a single-site type (Hill coefficients > 0.9). In addition, GTP (in the presence of dithiothreitol) had no appreciable effect on these displacement curves (results not shown). It was concluded that NECA only displayed 'antagonist-like' properties in these experiments. This would imply that binding of [3 H]DPCPX to any A_2 receptors in the membranes was negligible. Otherwise, NECA would have behaved like an agonist and a 'GTP shift' would have been observed. This suggests that we were selectively studying events at A_1 receptors. By contrast with PIA, the K_d for NECA in brown adipocyte membranes was similar to that observed in white adipocyte membranes (Table 1).

The effect of dithiothreitol was investigated further in the experiment shown in Fig. 6. As found with rat brain membranes (Lohse *et al.*, 1984), guanine nucleotides convert the saturation binding of [3 H]PIA into a low-affinity form. In accord with this, Gpp[NH]p decreased [3 H]PIA binding in white adipocyte membranes, particularly at low concentrations of the agonist. In the presence of dithiothreitol this effect of guanine nucleotides was accentuated.

In conclusion, white adipocyte lipolysis shows a modestly greater responsiveness to PIA compared with the process in brown adipocytes. This is paralleled by differences between the cell types in K_d values for PIA binding and for binding of the A_1 antagonist DPCPX. There may be a physiological rationale for brown adipocyte lipolysis being less responsive to adenosine. It remains to be fully established whether adenosine is a vasodilator in brown fat. If it were, then it would need to exert this vascular effect in thermogenic situations (where there is a large increase in blood flow) without switching off the lipolytic process that is fuelling thermogenesis. Further work would be necessary to

establish whether the data presented here could reflect the presence of different subtypes of the A₁ receptor in the two adipocyte types. In this regard, it is noteworthy that the brown adipose β -adrenergic receptor differs pharmacologically from that found in white adipose tissue (Arch *et al.*, 1984).

We have also found that dithiothreitol substantially enhances guanine nucleotide effects in the adipocyte membrane systems. At present it is not known whether this phenomenon may be observed in membranes from other tissues.

We are grateful to Mrs. C. Roberts and Mr. M. Orford for skilled technical assistance. This work was supported by a project grant from the Medical Research Council.

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Received 18 December 1989/9 March 1990; accepted 15 March 1990