Changes in the anti-lipolytic action and binding to plasma membranes of N^6-L-phenylisopropyladenosine in adipocytes from starved and hypothyroid rats

Paul CHOHAN, Carol CARPENTER and E. David SAGGERSON*

Department of Biochemistry, University College London, Gower Street, London WC1E 6BT, U.K.

1. The anti-lipolytic effect of the adenosine analogue N^6-L-phenylisopropyladenosine was studied with rat adipocytes incubated with a high concentration of adenosine deaminase (0.5 unit/ml, approx. 2.5 µg/ml) and concentrations of noradrenaline that were equieffective in different physiological states. 2. These studies were performed to compare the fed and starved (24 h) states and to compare a hypothyroid state (induced by feeding propylthiouracil + a low-iodine diet) with the euthyroid state. 3. Starvation increased sensitivity of the cells to the lipolytic action of noradrenaline, while decreasing sensitivity to the antilipolytic action of phenylisopropyladenosine. 4. Hypothyroidism resulted in decreased sensitivity to noradrenaline and increased sensitivity to phenylisopropyladenosine. 5. Studies of the binding of [³H]phenylisopropyladenosine to adipocyte plasma membranes indicated heterogeneity of binding sites or negative co-operativity in the binding. 6. Starvation did not change [³H]phenylisopropyladenosine binding to membranes, whereas hypothyroidism caused an unexpected decrease in both the number and affinity of the binding sites. 7. These observations are discussed in terms of the dual regulation of adipose-tissue lipolysis by lipolytic and anti-lipolytic agents.

It is generally accepted that stimulation of lipolysis in adipose tissue by lipolytic hormones (e.g. adrenaline, noradrenaline, corticotropin, glucagon) is brought about by receptor-mediated activation of adenylate cyclase, leading to cyclic AMP accumulation and activation of cyclic AMP-dependent protein kinase, which phosphorylates the hormone-sensitive lipase, resulting in increased activity of the process. In recent years it has become clear that this process is under dual regulation in that adenylate cyclase activity regulation is the balance between the effects of stimulating (lipolytic) and inhibiting (anti-lipolytic) agents (e.g. adenosine and E-series prostaglandins) (Rodbell, 1980; Londos *et al.*, 1981; Wolff *et al.*, 1981). Inhibition of adenylate cyclase (and hence lipolysis) is mediated by the A_1 (Van Calker *et al.*, 1979) or R_i (Londos *et al.*, 1980) adenosine receptor, which is coupled to the catalytic unit of adenylate cyclase by a guanine nucleotide-binding protein (N_i) that is distinct from that (N_S) which couples stimulatory receptors to the enzyme (Rodbell, 1980; Murayama & Ui, 1983; Olansky *et al.*, 1983). Intact adipose tissue or incubations of adipocytes contain sufficient amounts of adenosine to attenuate the stimulatory effects of lipolytic hormones on the system (Schwabe *et al.*, 1973; Schwabe & Ebert, 1974; Fain & Wieser, 1975; Fredholm, 1976; Fernandez & Saggerson, 1978; Ohisalo & Stouffer, 1979; Fredholm & Sollevi, 1981; Aitchison *et al.*, 1982).

There have been several reports that in starvation or diabetes the sensitivity to lipolytic stimulators is increased (Zapf *et al.*, 1977; Zumstein *et al.*, 1980; Honnor & Saggerson, 1980; Dax *et al.*, 1981; Chohan & Saggerson, 1982; Chatzipanteli & Saggerson, 1983). Conversely, in hypothyroidism, sensitivity to lipolytic stimuli is decreased (Correze *et al.*, 1974; Malbon *et al.*, 1978; Ohisalo & Stouffer, 1979; Goswami & Rosenberg, 1980).

In three physiological states, namely hypothyroidism (Ohisalo & Stouffer, 1979), adrenalectomy (Saggerson, 1980), and lactation compared with pregnancy (Vernon *et al.*, 1983), the response of the system to adenosine (or more correctly the analogue PIA) is increased. It has not been established whether these are post-receptor changes or are

attributable to alterations in the number or affinity of adenosine receptors.

The present study had two objectives. We have investigated the effect of brief starvation (24h) on the sensitivity of rat adipocyte lipolysis to PIA and find it to be substantially decreased. In addition we have measured the binding of [^{3}H]PIA to adipocyte plasma membranes to examine the characteristics of putative adenosine receptors under conditions where the sensitivity of lipolysis to this agent is decreased (starvation) or increased (hypothyroidism).

Materials and methods

Chemicals

Chemicals were obtained and treated as described by Honnor & Saggerson (1980) and Saggerson (1980). In addition, N^6-L-2-phenylisopropyl[2,8-^{3}H]adenosine was from Amersham International, Amersham, Bucks., U.K.

Animals

In all cases these were male Sprague–Dawley rats bred in the animal colony at University College London.

Animals used for comparison of the fed and the starved states received only GR3-EK cube diet (E. Dixon and Sons, Ware, Herts., U.K.) until selected for experiments at 160–180 g body wt. Starved animals had food removed 24h before use. Normal drinking water was always available.

Animals used for comparison of the euthyroid and the hypothyroid states were treated as follows. Approx. 4 weeks before use, the animals were taken off the GR3-EK diet and transferred to diets supplied by Special Diet Services, Witham, Essex, U.K. The euthyroid animals were fed on rat and mouse maintenance diet (no. 1, standard cube) and drank water containing 0.25% (v/v) ethanol. The hypothyroid animals were fed on an iodine-deficient version of the same diet and drank water containing 0.01% (w/v) 6-n-propyl-2-thiouracil. Ethanol was used as a vehicle to dissolve the propylthiouracil, and hence was also present in the drinking water at 0.25% (v/v). The animals selected to be made hypothyroid were approx. 10 days older than their euthyroid controls, in order to avoid too great a difference in body weight and fat-pad size at the end of the 4-week dietary treatment. The success of induction of the hypothyroid state was assessed by following both growth rates and changes in plasma concentrations of thyroid hormones. Expressed as means ± S.E.M., euthyroid rats weighed 76 ± 1 g ($n = 95$) when growth monitoring started and were 225 ± 3 g ($n = 92$) at the time of death 4 weeks later. Hypothyroid animals grew from 112 ± 1 g ($n = 100$) to 175 ± 2 g ($n = 99$)

over the same 4-week period. Hypothyroid animals exhibited negligible growth over the final week. Tri-iodothyronine and thyroxine concentrations in plasma at the time of death were determined at University College Hospital by radioimmunoassay and were found to be (means ± S.E.M.): euthyroid animals ($n = 21$), thyroxine = 32.6 ± 2.4 nM, tri-iodothyronine = 1.06 ± 0.04 nM; hypothyroid animals ($n = 18$), thyroxine ⩽ 10 nM (below the limits of the assay), tri-iodothyronine = 0.17 ± 0.01 nM.

Preparation and incubation of adipocytes

Adipocytes were prepared from epididymal fat-pads essentially as described by Rodbell (1964). For studies of lipolysis, adipocytes equivalent to one-sixteenth of a fat-pad were incubated at 37°C in plastic vials containing 1.0 ml of Krebs–Henseleit (1932) saline, 4% (w/v) fatty acid-poor albumin and 5 mM-glucose. The gas phase was O_2/CO_2 (19:1). After 60 min, the contents of the vials received $HClO_4$ to a final concentration of 6% (w/v) and were then neutralized and treated as described by Fernandez & Saggerson (1978). These extracts were assayed for glycerol by the method of Garland & Randle (1962). Where adenosine deaminase (EC 3.5.4.4) was added to adipocyte incubations, the enzyme was previously dialysed overnight against 0.9% NaCl to remove $(NH_4)_2SO_4$, followed by spectrophotometric standardization at 265 nm and 25°C by the method of Kalckar (1947). A unit of adenosine deaminase is the amount of enzyme needed to deaminate 1 μmol of adenosine/min at 25°C in this assay.

Preparation of adipocyte plasma membranes

Plasma membranes were prepared from epididymal adipocytes of five to eight rats. The cells were broken by agitation on a vortex mixer in 15 ml of ice-cold 0.25 M-sucrose/10 mM-Tris/HCl/2 mM-EGTA, pH 7.4, and rapidly fractionated by the method of Belsham et al. (1980), which employs density-gradient centrifugation in Percoll. Finally, the membrane preparation was suspended in 50 mM-Tris/HCl buffer (pH 7.4) containing 1 mM-$MgCl_2$ ('Tris–Mg buffer'). The protein content was determined (Lowry et al., 1951) with bovine albumin as a standard, and the membranes were stored in liquid N_2 at a protein concentration of 1–2 mg/ml. These preparations exhibited similar activities and cross-contamination by marker enzymes to those reported by Chohan & Saggerson (1982).

Measurement of [^{3}H]PIA binding to plasma membranes

The method of Trost & Schwabe (1981) was used with some modifications. In essence, the mem-

branes were obtained by the more rapid Percoll-based fractionation; these were treated with a larger amount of adenosine deaminase to remove endogenous adenosine, and binding assays were performed with substantially lower membrane protein concentrations.

Plasma membranes in Tris–Mg buffer (0.20 mg of protein/ml) were preincubated with freshly standardized, dialysed, adenosine deaminase (3 units/ml) for 10 min at 30°C before being used immediately in binding assays. These contained 70 μg of membrane protein incubated at 37°C for 15 min in 0.4 ml of Tris–Mg buffer containing the required concentration of [^{3}H]PIA. After incubation the membranes were separated from the medium by vacuum filtration through Whatman GF/B glass-fibre filters. The filters were washed with 2 × 5 ml of ice-cold Tris–Mg buffer and then counted for radioactivity in a scintillation fluid consisting of 2,5-bis-(5-t-butylbenzoxazol-2-yl)-thiophen (4 g/l) in toluene/Triton X-100 (2 : 1, v/v). Correction of non-specific binding was applied to all samples. This was determined by measurement of retained radioactivity when binding assays were performed in the presence of 0.1 mM unlabelled PIA. The radioactivity (c.p.m.) attributable to non-specific binding was proportional to the [^{3}H]PIA concentration over the range 0.1–150 nM, and was generally less than 20% and never more than 50% of the total measured in any sample. Specific binding was constant after a 15 min incubation, implying attainment of equilibrium. Binding was also proportional to membrane protein concentration.

Results and discussion

Effect of starvation on the anti-lipolytic action of PIA

This response of adipocytes can be assessed if endogenous adenosine is removed by addition of a high concentration of adenosine deaminase, followed by measurement of the ability of the adenosine analogue PIA (which is not metabolized by adenosine deaminase) to inhibit noradrenaline-stimulated lipolysis (Ohisalo & Stouffer, 1979; Saggerson, 1980; Vernon et al., 1983). Several studies have shown that adenosine deaminase alone, at concentrations comparable with or less than those used here (0.5 unit/ml, equivalent to approx. 2.5 μg of enzyme) increases basal lipolysis severalfold (Schwabe & Ebert, 1974; Fain & Wieser, 1975; Fernandez & Saggerson, 1978; Aitchison et al., 1982). However, in other studies high concentrations of adenosine deaminase had little effect on basal lipolysis, although the removal of endogenous adenosine did substantially facilitate the actions of lipolytic hormones (Ohisalo & Stouffer, 1979; Shechter, 1982). This was the

case in the present study. Adenosine deaminase (0.5 unit/ml) only increased basal lipolysis by 1.3- or 1.6-fold in cells from fed or starved rats respectively. These increases were not statistically significant. Noradrenaline (1 μM) in all cases caused a further 10–15-fold increase in lipolysis over this 'basal' rate, and therefore the effects of PIA reported below are almost entirely due to opposition of 'noradrenaline-stimulated' lipolysis rather than 'adenosine deaminase-stimulated' lipolysis. The reason for the variability in the lipolytic effects of adenosine deaminase between various studies is unexplained at present, but could be attributable to differences in strains of rats, seasonal variation or differences in cell concentrations in incubations. The effectiveness of the anti-lipolytic action of adenosine or of PIA against noradrenaline-stimulated lipolysis is dependent on the noradrenaline concentration (Stock & Prilop, 1974; Fredholm, 1978). No anti-lipolytic effects of the nucleosides are observed when relatively high (i.e. in the μM range) concentrations of noradrenaline are used. It is therefore important to choose sub-maximal concentrations of the stimulating agent. Furthermore, for comparisons between physiological states it is also important that concentrations should be chosen that are at equivalent points on the noradrenaline-dose-response curve. Since prolonged (72 h) starvation (Zapf et al., 1977; Dax et al., 1981) or experimental diabetes (Zumstein et al., 1980) result in 3–5-fold increases in sensitivity of lipolysis to adrenaline, preliminary experiments were performed in order to choose appropriate concentrations for this study. The values given are means±s.e.m. from three separate experiments. Previous findings (Zapf et al., 1977; Dax et al., 1981) were confirmed in that half-maximal stimulation of lipolysis was achieved with 16±4.5 nM- and 2.2±1.3 nM-noradrenaline in the fed and starved states respectively ($P < 0.05$), whereas 160±31 nM and 45±10 nM concentrations elicited 75% of the maximal lipolytic response ($P < 0.05$). Accordingly, these noradrenaline concentrations were used to investigate the anti-lipolytic action of PIA in the fed and starved states (Fig. 1). In the fed state PIA showed a significant ($P < 0.025$) anti-lipolytic effect at all tested concentrations against the half-maximal dose of noradrenaline. With the 75% maximally effective dose of noradrenaline, only 1 mM-PIA caused significant inhibition of lipolysis in the fed state. Starvation for 24 h resulted in considerable loss of sensitivity to PIA in the concentration range 0.1 nM–0.1 mM.

Binding of [^{3}H]PIA to plasma membranes from fed and starved rats

Saturable specific binding of [^{3}H]PIA was

observed with plasma membranes from both fed and starved rats (Fig. 2a). PIA binding was slightly greater in the starved state at all the tested concentrations (0.1–120nM). This increase was not statistically significant (Student's *t* test). Scatchard
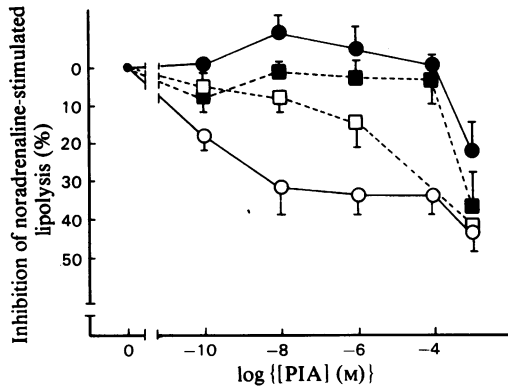

Fig. 1. *Inhibition by PIA of lipolysis in adipocytes from fed* (○, □) *and 24h-starved* (●, ■) *rats*
Incubation was for 60min with adenosine deaminase (0.5 unit/ml) and sufficient noradrenaline to elicit either 75% (broken lines) or 50% (continuous lines) of maximal lipolysis. For fed rats these were 160nM- and 16nM-noradrenaline respectively. For the cells from starved animals these noradrenaline concentrations were 45nM and 2.2nM respectively. The values are means±s.E.M. for four separate experiments.

analysis of the meaned data for both states (Fig. 2b) gave curvilinear relationships, suggesting either the presence of more than one class of binding site or negative co-operativity in the binding. Hill coefficients were 0.91 ± 0.08 and 0.78 ± 0.09 and half-maximal binding was observed with 3.2 ± 1.0nM- and 4.8 ± 1.4nM-PIA in the fed and starved states respectively (means ±s.E.M. in every case). Maximum PIA binding per mg of membrane protein in the fed state (680fmol/mg) was approx. 45% of the value obtained by Trost & Schwabe (1981), who studied PIA binding to adipocyte plasma membranes from fed rats over the concentration range 2–70nM. Their data yielded a linear Scatchard plot, and it was therefore concluded that there was a single class of PIA-binding site ($K_D = 6.1$ nM). The insert in Fig. 2(b) shows that similar treatment of our data for the fed state (i.e. consideration only of the data points for PIA concentrations $\geqslant 2$nM) also yields a linear Scatchard plot ($K_D = 3.2$nM). However, this ignores the presence of higher-affinity binding, which is seen with PIA concentrations $\leqslant 1.5$nM. In the starved state the curvature of the Scatchard plot was more pronounced, and attempts to fit a straight line to any portion were fruitless. These data were not analysed further, but led to the conclusion that the starvation-induced decrease in sensitivity to PIA (Fig. 1) could not readily be attributed to alteration in the binding parameters at adenosine receptors.
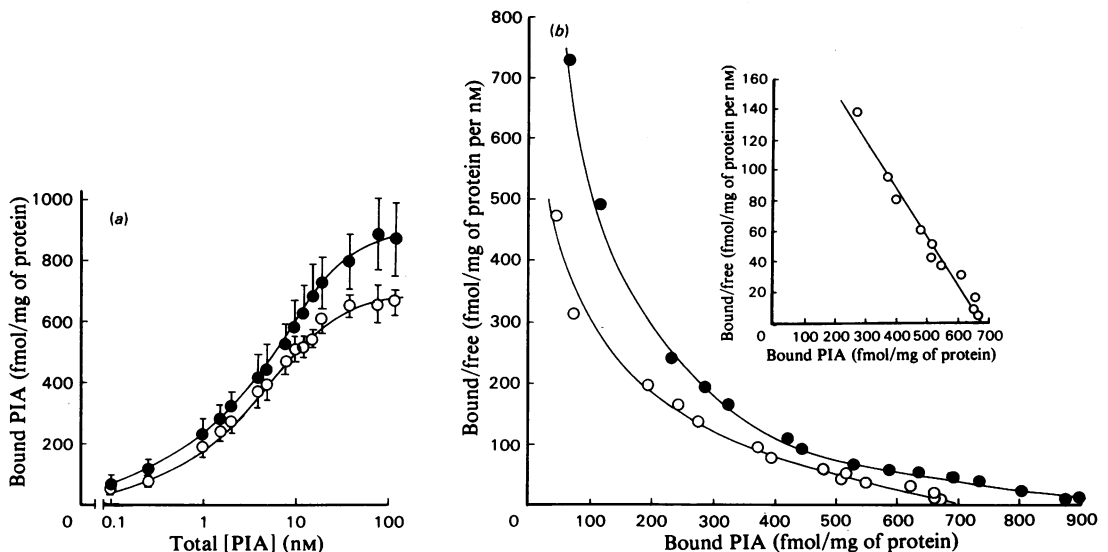

Fig. 2. *Binding of* [³H]*PIA to adipocyte plasma membranes from fed* (○) *and 24h-starved* (●) *rats*
(a) The values are means±s.E.M. for five separate experiments. (b) Scatchard analysis of the same data. The inset plot (*r* = 0.98) represents Scatchard analysis of the data for the fed state, only considering values where [PIA] $\geqslant 2$nM. This latter treatment incorrectly suggests the existence of a single class of binding sites with $K_D = 3.2$nM and maximum binding capacity = 680fmol/mg of protein.

Effect of hypothyroidism on the anti-lipolytic action of PIA

The experiment summarized in Fig. 3 was performed to assess whether our animal model of hypothyroidism showed increased sensitivity to PIA, as previously reported by Ohisalo & Stouffer (1979). Preliminary experiments demonstrated that cells from the hypothyroid animals were slightly less sensitive to noradrenaline even with a high concentration of adenosine deaminase present. In the euthyroid state 75% of maximal lipolysis was seen with 170 ± 23 nM-noradrenaline. With cells from hypothyroid animals, 480 ± 99 nM-noradrenaline was required to cause 75% of maximal lipolysis ($n = 3$ in both states, $P < 0.05$). Fig. 3 demonstrates that the cells from the euthyroid animals were virtually insensitive to PIA when the 75%-maximally-effective noradrenaline concentration was present. A considerable increase in response to PIA was seen in the hypothyroid state. Concentrations of PIA $\leqslant 0.1$ nM caused a 50% inhibition of lipolysis elicited by the 75%-maximally-effective dose of noradrenaline, and the degree of inhibition of lipolysis was substantially increased.

Binding of [³H]PIA to plasma membranes from euthyroid and hypothyroid rats

Adipocyte plasma membranes were obtained from the hypothyroid animals which showed this considerable increase in sensitivity to PIA. [³H]PIA binding was studied in the expectation

Fig. 3. *Inhibition by phenylisopropyladenosine of lipolysis in adipocytes from euthyroid* (○) *and hypothyroid* (●) *rats* Incubation was for 60 min with adenosine deaminase (0.5 unit/ml) and sufficient noradrenaline to elicit 75% of maximal lipolysis. For euthyroid rats this was 170 nM-noradrenaline. For the cells from hypothyroid animals this was 480 nM. The values are means $\pm$ S.E.M. for four separate experiments.

that these preparations would show increased and/or tighter binding of PIA compared with membranes from euthyroid animals. Although saturable specific binding was seen in both states (Fig. 4a), the affinity of binding was clearly decreased, with a lower total number of binding sites in the hypothyroid state. PIA binding was significantly less in the hypothyroid state at all tested concentrations ($P < 0.001$ for PIA concentrations of 0.1–20 nM and $P < 0.05$ for higher concentrations). It is noteworthy that the maximum binding in the membranes from the euthyroid animals was appreciably greater than that with those from fed animals in Fig. 2(a). This can only be attributed to differences in the feeding routines, since both sets of rats were obtained from the same rat population and both sets of experiments were performed over the same time period. Scatchard analysis of the means of these data (Fig. 4b) again suggested either negative co-operativity in the PIA binding or the existence of more than one class of site. Hill plots were linear, giving Hill coefficients of 0.60 ± 0.03 and 0.87 ± 0.03, with half-maximal binding at 3.9 ± 0.6 nM- and 12.5 ± 1.1 nM-PIA in the euthyroid and hypothyroid states respectively (means $\pm$ S.E.M.; $P < 0.001$ for both sets of measurements). The higher-affinity component of the binding was quantified in two ways. First, linear regression analysis was applied to those portions of the Scatchard plots showing a clear linear correlation (the first seven points for euthyroid rats and the first nine for hypothyroid rats). This approach gives maximum estimates for the binding capacity and K_D at the higher-affinity sites. These values are stated in the legend to Fig. 4(b). In addition, these data were analysed by a non-linear least-squares-fitting computer program, FACSIMILE (Chance et al., 1977), describing two independent classes of binding sites for each set of meaned data. This yielded estimates ($\pm$ S.D.) for K_D and binding capacity (n) at the higher-affinity sites of: euthyroid, $K_D = 0.28 \pm 0.02$ nM, $n = 530 \pm 29$ fmol/mg of protein; hypothyroid, $K_D = 1.14 \pm 0.23$ nM, $n = 145 \pm 39$ fmol/mg of protein. Unfortunately binding parameters for the lower-affinity sites were less well determined. Clearly, whichever form of analysis is used, it must be concluded that the hypothyroid state results in a decreased-affinity state of the adenosine receptor as measured in isolated plasma membranes. Again, it must be considered that change in sensitivity to PIA (Fig. 3) cannot readily be attributed to alterations in the receptor binding parameters.

General discussion

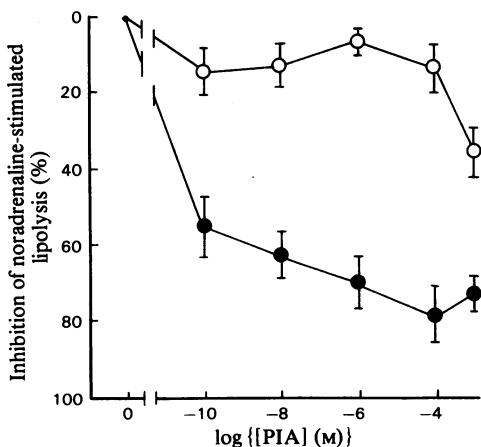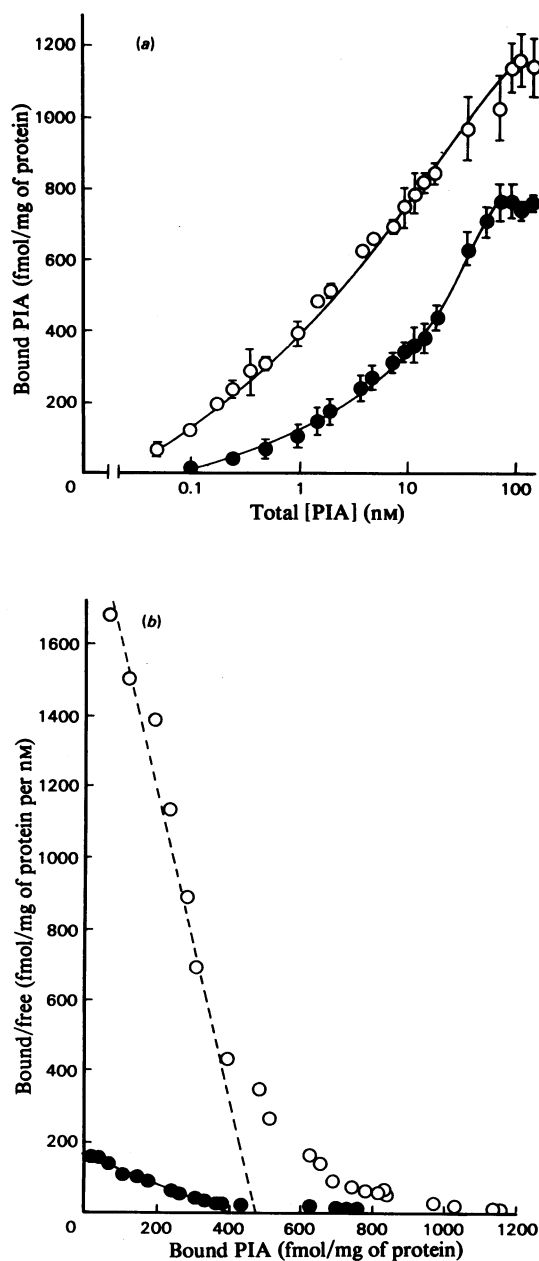It is apparent from this study that the responsiveness of both the stimulatory and inhibitory

Fig. 4. *Binding of [³H]PIA to adipocyte plasma membranes from euthyroid* (○) *or hypothyroid* (●) *rats*

(*a*) The values are means of five separate experiments. The bars indicate s.e.m.; where not shown, these lie within the symbol. (*b*) Scatchard analysis of the same data. For euthyroid rats, the broken line ($r = 0.98$) suggests a set of tight-binding sites with $K_D \leqslant 0.24$nM and maximum binding capacity of $\leqslant 490$fmol/mg of protein. For hypothyroid rats these values are $K_D \leqslant 2.4$nM and maximum binding capacity $\leqslant 390$fmol/mg of protein ($r = 0.99$ for the regression line).

sides of the adenylate cyclase/lipolysis dual regulation system may be changed in opposite directions. These effects appear to be separate, i.e. changes in sensitivity to PIA were seen in the presence of equieffective concentrations of noradrenaline, and changes in sensitivity to noradrenaline were seen in the presence of a high concentration of adenosine deaminase to remove endogenous adenosine. Lipolysis could therefore be increased in starvation by a combination of factors, i.e. by an increased concentration of lipolytic agent(s), by increased sensitivity to these stimulating agents and also by decreased responsiveness to endogenous or 'local' anti-lipolytic factors such as adenosine.

Changes in sensitivity to PIA were not accompanied by parallel changes in the affinity or capacity of binding of this agent to plasma membranes. Indeed, substantial changes in the opposite direction were observed in hypothyroidism. In this respect it is noteworthy that altered sensitivity of the system to stimulation by catecholamine hormones in hypothyroidism or starvation is reported as not being accompanied by any changes in the K_D or number of β-adrenoceptors (Malbon *et al.*, 1978; Goswami & Rosenberg, 1980; Dax *et al.*, 1981). Other studies of these physiological states have observed alterations in β-adrenoceptor number without changes in K_D (Giudicelli, 1978; Giudicelli *et al.*, 1982).

The [³H]PIA-binding studies suggest a higher affinity of binding to the adipocyte membrane than hitherto reported. Furthermore, there appears to be heterogeneity in the adenosine-binding sites. This is also seen in other systems, i.e. two binding sites have been observed in studies of 2-chloroadenosine binding to rat brain membranes (Williams & Risley, 1980) and N^6-cyclohexyladenosine binding to bovine membranes (Bruns *et al.*, 1980). More recently Siu-Mei & Green (1983) have reported the existence of three affinity states of the adenosine receptor in membrane preparations from rat hippocampus. It is of interest to establish which components of this heterogeneous binding may be functionally linked with the control of the adenylate cyclase/lipolysis system in the adipocyte.

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