HETEROGENEITY AND SUBCELLULAR LOCALIZATION OF HAMSTER ADIPOCYTE α-ADRENERGIC RECEPTORS

Evidence of α_1-and α_2-subtypes

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1. Introduction

Hamster white fat cells are known to display, like the human adipocytes [1], a mixed α- and β-adrenergic sensitivity [2,3], which set these cells apart from the more frequently used and almost exclusively β-sensitive rat white adipocytes [4]. Methods measuring the binding of the labeled antagonist [^{3}H]-dihydroergocryptine have been successfully used for the direct identification of α-adrenergic receptors in different tissues [5–8]. Applying these binding techniques to a 'crude' membrane fraction prepared from hamster adipocytes, we have identified and characterized specific binding sites for [^{3}H]DHEC in this preparation [9]. Although most of the characteristics expected of true physiological α-adrenergic receptors were fulfilled by these binding sites [9], some of their properties suggested that these sites may be heterogeneous or that only a part of them may represent the true α-receptor sites.

This has led us to reexamine in detail some of the properties of these [^{3}H]DHEC binding sites. We report here the subcellular localization of these sites, the results of competition experiments using two highly selective α_1- and α_2-adrenergic antagonists, prazosin and yohimbine [10–11] and finally binding data obtained with membranes treated under conditions shown to induce an irreversible block of the fat cell physiological α-responsiveness. We show that [^{3}H]DHEC binding sites are almost exclusively localized in the adipocyte plasma membrane and are made up of two components which can be classified at least in part as α_1- and α_2-receptor subtypes.

Abbreviations: DHEC, dihydroergocryptine; cyclic AMP, adenosine-3′:5′-monophosphate

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2. Experimental

2.1. *Chemicals*

[^{3}H]DHEC (spec. act. 22 Ci/mmol) was obtained from the Radiochemical Centre (Amersham). Fresh stock solutions were prepared and diluted for the binding assays as in [9]. Prazosin and clonidine were gifts from Pfizer (France) and Boehringer (Ingelheim), respectively. Yohimbine, (–)-epinephrine, (–)-norepinephrine, and dopamine were from Sigma. Cyclic [^{3}H]AMP and cyclic AMP-binding protein were purchased from the Radiochemical Centre, Amersham. The sources of all the other reagents have been described [9]. Fresh stock solutions of phentolamine, phenoxybenzamine, prazosin, yohimbine and clonidine were prepared as in [6,9,11].

2.2. *Preparation of crude membranes and subcellular fractions*

Epididymal fat pads from fed golden hamsters (Charles River) 95–105 g, were pooled and isolated fat cells prepared as in [3]. Isolated fat cells were resuspended in medium I (0.25 M sucrose, 1 mM EDTA, 10 mM Tris–HCl, pH 7.4) and disrupted according to [12]. After breakage, the suspension was centrifuged, washed 3 times and resuspended as in [13]. The final pellet (crude membrane) was resuspended in medium II (10 mM $MgCl_2$, 50 mM Tris–HCl) resulting in a suspension containing

2–2.5 mg protein/ml which was used in binding assays. Adipocyte plasma membranes, mitochondrial and microsomal membrane preparations were prepared as in [14]. These fractions were resuspended in medium II, stored for 24 h in liquid nitrogen and assayed for [^{3}H]DHEC binding.

2.3. *Binding assay*

Binding assays were done by a slight modification of the method in [6]. Protein (200 μg) was usually incubated with [^{3}H]DHEC in 150 μl total vol. of medium II for 10 min with shaking at 37°C. Incubations were terminated by adding 5 ml incubation buffer, followed by a rapid vacuum filtration of the suspension through a Whatman GFC glass fiber filter. Filters were rapidly washed with 15 ml buffer. The filtration and wash steps were completed in <30 s. Filters were dried, added to 10 ml scintillation cocktail (MI 96, Packard) and counted with an efficiency of 40%. Non-specific binding was determined by measuring the radioactivity retained on filters when incubations were performed with a large excess (10 μM) of phentolamine. All values refer to specific binding which averaged 75–85% of the total counts bound and which was defined as total radioactivity bound minus non-specific binding. As attested by chromatographical studies [6], no degradation of the radiolabeled ligand could be detected during the binding incubations.

2.4. *Other determinations*

Incubation of fat cells and determination of cyclic AMP were done as in [3,15]. Protein was determined according to [16].

3. Results and discussion

The subcellular distribution of [^{3}H]DHEC binding sites in hamster adipocytes was studied by measuring the amount of [^{3}H]DHEC which specifically binds to fractions enriched in plasma membranes, mitochondria and microsomes, when these fractions are incubated for 10 min at 37°C in the presence of 5 nM [^{3}H]DHEC. Of the fractions tested, binding was predominantly localized in the plasma membrane fraction. Indeed, taking into account the recovery of each fraction, specific binding of [^{3}H]DHEC was undetectable in the mitochondrial fraction whereas in microsomes it was <15% of the specific binding found in the plasma membrane fraction (not shown). As documented by 5′-nucleotidase analysis and as pointed out [17], the small amount of [^{3}H]-DHEC binding to the microsomal fraction was probably accounted for by some contamination of this fraction with plasma membranes. Thus, and because the preparation of the plasma membrane fraction is a multistep process requiring several hours, all the following experiments were performed on microsome-free crude membranes.

Studies concerned with α-adrenergic receptors in brain [11,12], heart [18] and smooth muscle [19,20] have clearly shown that [^{3}H]DHEC could bind in these tissues to two different types of sites. To determine whether such a situation could also apply to hamster fat cells, inhibition of [^{3}H]DHEC binding by different α-adrenergic antagonists and agonists were studied and the resulting data transformed as log–logit plots. As shown in table 1, the α-antagonists phentolamine and phenoxybenzamine potently competed for the [^{3}H]DHEC binding sites with respective EC_{50} values of 120 and 180 nM; the same applied for the α-agonists (–)-epinephrine (EC_{50} = 400 nM) and (–)-norepinephrine (EC_{50} = 600 nM). In contrast, dopamine and serotonine were weak competitors (EC_{50} = 100 and 300 μM, respectively). Log–logit plots derived from these displacement curves consistently displayed slopes less

Table 1
Inhibition by various drugs of the specific [^{3}H]dihydroergocryptine binding to hamster adipocyte membranes

Drugs	EC_{50} (nM)	Log–logit slopes
(–)-Epinephrine	400	0.48
(–)-Norepinephrine	600	0.55
Phentolamine	120	0.60
Phenoxybenzamine	180	0.48
Dopamine	100 000	0.71
Serotonine	300 000	0.75

Hamster adipocyte membranes were incubated with [^{3}H]-DHEC (8.5 nM) alone or in combination with increasing concentrations of the indicated drugs. After 10 min incubation, the amount of [^{3}H]DHEC remaining specifically bound was determined. Log–logit plots were drawn and slope values calculated by regression analysis. EC_{50} values refer to the concentration of each drug causing 50% inhibition of [^{3}H]DHEC binding. Values are mean of 2 expt with binding at each drug concentration assayed in duplicate in each experiment

than unity to suggest displacement by these compounds at two different [^{3}H]DHEC binding sites (table 1).

This was confirmed by the following experiments in which the steady-state binding of [^{3}H]DHEC to hamster adipocyte membranes was studied as a function of [^{3}H]DHEC concentration. As shown in fig.1, the [^{3}H]DHEC saturation binding curve was apparently biphasic with a break at ~3 nM. A Scatchard analysis [21] of these data (fig.1, inset) suggested two straight lines consistent with the existence of two orders of [^{3}H]DHEC binding sites. One set of binding sites had high affinity (K_d 2 nM) but low binding capacity (30% of the total [^{3}H]DHEC binding sites), whereas the other set of sites displayed low affinity but high binding capacity and possibly apparent positive cooperativity (K_d 11 nM, B_{max} = 0.68 pmol/mg protein, Hill number = 1.4, values calculated after subtraction of the contribution of the high affinity sites). These results are thus different from the data in [9] where a single homogeneous class of binding sites was reported. This discrepancy appears related to the fact that in the former study, the binding of [^{3}H]DHEC at low ligand concentrations was determined from a more limited number of [^{3}H]DHEC concentrations than here, thus rendering the data inaccurate.

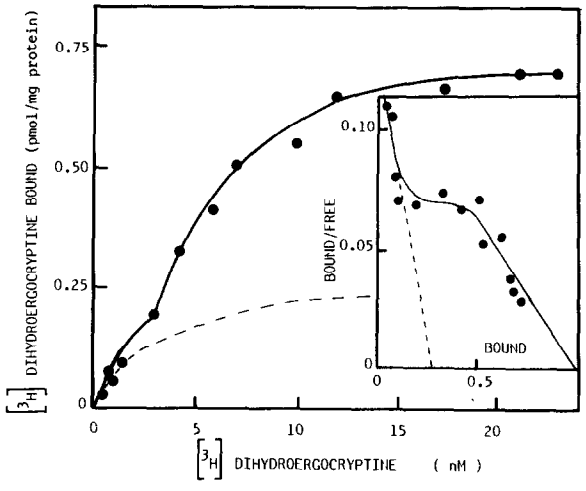

Fig.1. Specific binding of [^{3}H]DHEC to hamster adipocyte membranes as a function of [^{3}H]DHEC concentration. Hamster adipocyte membranes were incubated with the indicated concentrations of [^{3}H]DHEC and specific binding was determined as in section 2. Each value is the mean of 3 determinations from representative experiment, each experiment being repeated 3 times. Inset: Scatchard plot of [^{3}H]DHEC binding.

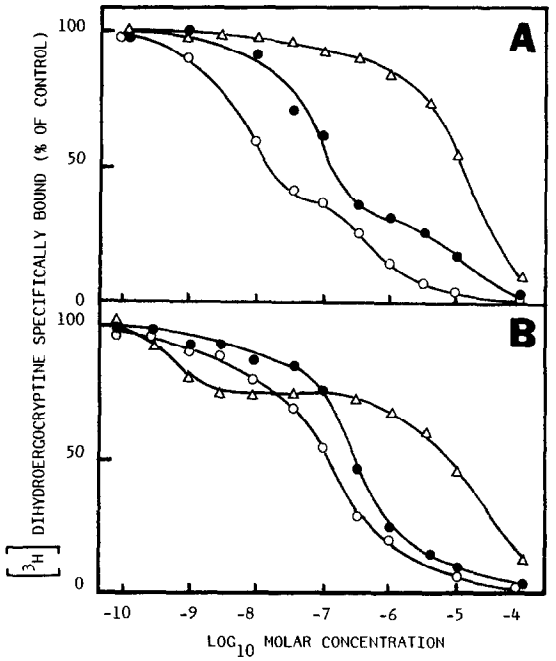

Fig.2. Displacement of [^{3}H]DHEC bound to hamster adipocyte membranes by α-adrenergic agonist and antagonists. Membranes (200 μg protein) were incubated at 37°C with 2.5 nM (A) or 8.5 nM (B) [^{3}H]DHEC in the absence (control) or presence of various concentrations of clonidine (○), yohimbine (●) or prazosin (△). After 10 min incubation, the amount of [^{3}H]DHEC remaining bound was determined as in section 2. Each point is the mean of 3 determinations from one representative experiment, each experiment being repeated at least twice.

For further analysis, we studied at two different [^{3}]DHEC concentrations, the displacement of this ligand by two selective α_1- and α_2-antagonists, respectively, prazosin and yohimbine [11,12,18,19] and by one α_2-agonist, clonidine [22]. At 2.5 nM [^{3}H]DHEC, a concentration labeling predominantly the apparent high affinity [^{3}H]DHEC binding sites (fig.1), yohimbine and clonidine showed similar biphasic competition curves (fig.2A) suggesting the existence of two classes of binding sites for each of these drugs: in fact, both of these drugs initially displaced with high affinity 60–70% of the labeled sites and displaced only with low affinity the remaining binding sites. In contrast, prazosin was a weak competitor under these conditions. From fig.2A, it appears likely that the 70% of the labeled sites which have quite high affinity for clonidine and yohimbine are presumably α_2 by nature, although the lower affinity sites for

yohimbine and clonidine are hard to classify since their affinity is also higher than that of prazosin. At 8.5 nM [^{3}H]DHEC, at which 60–70% of the labeled sites represent the low affinity [^{3}H]DHEC binding sites, the competition curves for yohimbine and clonidine were apparently monophasic (fig.2B); under the same conditions, prazosin showed a sharp biphasic competition curve indicating the existence of two apparent orders of sites, one with high affinity representing 25% of the labeled sites (maximal competition between 3 and 300 nM) and one with low affinity representing the 75% remaining sites. Thus, the bulk of sites (70–75%) which have much higher affinity for yohimbine and clonidine than for prazosin are clearly α_2. So the proportion of α_2 receptor subtype is about the same whether the [^{3}H]DHEC binding sites to be displaced are predominantly the high or the low affinity [^{3}H]DHEC binding sites. This is not surprising considering reports showing that [^{3}H]DHEC labels α_1-and α_2-receptors with equal affinity in tissues other than adipose tissue (reviewed [23]).

To have a better estimation of the respective proportions of α_1 and α_2 receptors, experiments were performed in which we compared the Scatchard plots of [^{3}H]DHEC binding in the absence and in the presence of 100 nM prazosin, a concentration which saturates the high affinity binding component of this antagonist in the competitive experiments depicted in fig.2B. As shown in fig.3, maximal binding was reduced by 17% in the presence of prazosin. Thus, these results provide pharmacological evidence that the [^{3}H]DHEC binding sites of hamster fat cell membranes are a mixed population of α_1- (17%) and

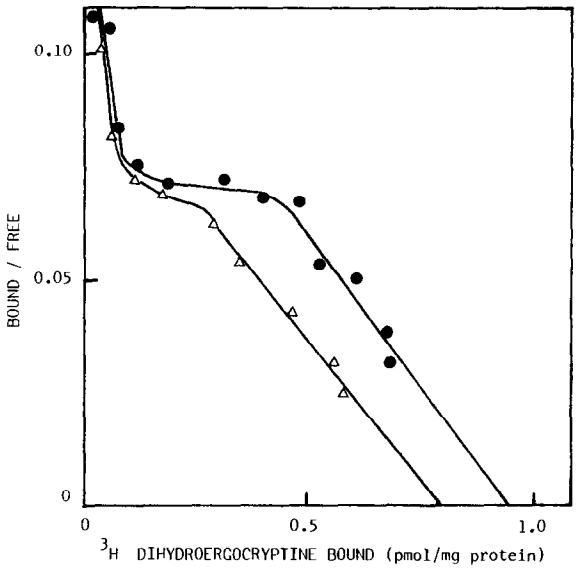

Fig.3. Scatchard plot of the concentration-dependent binding of [^{3}H]DHEC in the presence of prazosin. Hamster adipocyte membranes were incubated with [^{3}H]DHEC as in fig.1, in the absence (•) or presence of 100 nM prazosin (△). Each point is the mean of 3 determinations from 1 expt.

α_2- (83%) receptors subtypes, a possibility which has been evoked in earlier physiological studies on the role of α-adrenergic receptors in the control of glucose metabolism in these cells [24].

In order to test the physiological significance of both these high and low affinity [^{3}H]DHEC binding sites, the binding of [^{3}H]DHEC was studied in membranes preexposed to conditions inducing an irreversible block of the α-adrenergic responsiveness of the intact cells. These conditions consisted in incubating

Table 2
Influence of a preliminary incubation with phenoxybenzamine on the α-adrenergic responsiveness of hamster fat cells

Preincubation	Cyclic AMP produced (nmol . g lipid^{-1} . 30 min^{-1}) in response to	
	Epinephrine (50 μM)	Epinephrine (50 μM) + Phenoxybenzamine (100 μM)
Control	9.99 ± 0.52	18.70 ± 1.43
Phenoxybenzamine (100 μM)	22.64 ± 3.92 ($P < 0.001$)	21.50 ± 1.97 ($P > 0.1$)

Fat cells were preincubated at 37°C in the absence (control) or presence of phenoxybenzamine (100 μM). After 30 min, cells were repeatedly washed and reincubated with epinephrine alone or in combination with phenoxybenzamine. After 30 min, total cyclic AMP (cells + medium) was determined. Each value is the mean ± SE of 4 incubations

fat cells for 30 min at 37°C with 100 μM (the concentration eliciting the maximal rise in the epinephrine-induced cyclic AMP production [unpublished]) of phenoxybenzamine, an α-antagonist known to block irreversibly the α-adrenergic receptors in different tissues [20,25,26]. After repeated washing, such pretreated fat cells had lost their α-adrenergic responsiveness since the cyclic AMP produced by these cells after a subsequent incubation with epinephrine (50 μM) alone or in combination with phenoxybenzamine (100 μM) were not only equivalent but also identical with the cyclic AMP produced by epinephrine plus phenoxybenzamine in control cells (preincubated in the absence of phenoxybenzamine) (table 2). These experimental conditions were thus applied to adipocyte membranes which were first incubated with 100 μM phenoxybenzamine, repeatedly washed and subsequently assayed for [^{3}H]DHEC binding. Under these conditions, specific binding of [^{3}H]DHEC to both the high and low affinity sites was completely suppressed in these membranes (not shown), indicating that these [^{3}H]DHEC binding sites do, in fact, represent at least in part the functional α-receptors of the hamster fat cells.

The high potency of clonidine and yohimbine to displace most of the specific [^{3}H]DHEC binding sites would be an argument in favour of the presynaptic character of these α_2-binding sites [27]. This seems, however, highly unlikely because of the nature of the membranous preparation used which was not directly obtained from adipose tissue homogenates but from neural tissue-free fat cells. Therefore hamster fat cells can be considered as an additional example of a non-neural tissue [19,23] in which a post-synaptic α_2-receptor subtype can be identified by radio-ligand binding studies.

A relationship between binding data and physiological α-adrenergic responses provides strong evidence in favour of the existence of functional α_1- and α_2-adrenergic receptor subtypes in hamster fat cell membranes. Experiments using [^{3}H]norepinephrine, [^{3}H]prazosin and [^{3}H]clonidine are currently underway to determine the affinities of these receptors, their metabolic role and their possible hormonal regulation which is suggested by our observation that both the α-adrenergic responsiveness and the number of specific [^{3}H]DHEC binding sites of hamster fat cells are variable with age and cell size (unpublished) and are decreased by thyroid hormones [28]. Such experiments would also be helpful in the understanding of the molecular mechanisms which underlie α-receptor mediated adrenergic stimulation, namely the effects linked to the binding of catecholamine to α-receptors and the role played by calcium [29,30].

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References

[1] Burns, T. W., Langley, P. E. and Robison, C. A. (1971) Ann. NY Acad. Sci. 185, 115–128.
[2] Hittelman, K. J., Wu, C. F. and Butcher, R. W. (1973) Biochim. Biophys. Acta 304, 188–196.
[3] Giudicelli, Y., Agli, B., Brulle, D. and Nordmann, R. (1977) FEBS Lett. 83, 225–230.
[4] Stock, K. and Thomas, T. (1975) Metabolism, 24, 277–286.
[5] Williams, L. T. and Lefkowitz, R. J. (1976) Science 192, 791–793.
[6] Williams, L. T., Mullikin, D. and Lefkowitz, R. J. (1976) J. Biol. Chem. 251, 6915–6923.
[7] Greenberg, D. A. and Snyder, S. H. (1977) Life Sci. 20, 927–931.
[8] Kafka, M. S., Tallman, J. F. and Smith, C. C. (1978) Life Sci. 21, 1429–1438.
[9] Pecquery, R., Malagrida, L. and Giudicelli, Y. (1979) FEBS Lett. 98, 241–246.
[10] Peroutka, S. J., Greeberg, D. A., U'Prichard, D. C. and Snyder, S. H. (1978) Mol. Pharmacol. 14, 403–412.
[11] Miach, P. J., Dausse, J. P. and Meyer, P. (1978) Nature 274, 492–494.
[12] Avruch, J. and Wallach, D. F. H. (1971) Biochim. Biophys. Acta 233, 334–347.
[13] Giudicelli, Y. and Pecquery, R. (1978) Eur. J. Biochem. 90, 413–419.
[14] Khoo, J. C., Jarett, L., Mayer, S. E. and Steinberg, D. (1972) J. Biol. Chem. 247, 4812–4818.
[15] Giudicelli, Y., Pecquery, R., Provin, D., Agli, B. and Nordmann, R. (1977) Biochim. Biophys. Acta 486, 385–398.
[16] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.

[17] Jarett, L. (1974) Methods Enzymol. 31/A, 60.
[18] Guicheney, P., Garay, R. P., Levy-Marchal, C. and Meyer, P. (1978) Proc. Natl. Acad. Sci. USA 75, 6285–6289.
[19] Hoffman, B. B., De Lean, A., Wood, C. L., Shoken, D. D. and Lefkowitz, R. J. (1979) Life Sci. 24, 1739–1746.
[20] Kunos, G., Hoffman, B. B., Kwok, Y. N., Kan, W. H. and Mucci, L. (1979) Nature, 278, 254–256.
[21] Scatchard, G. (1949) Ann. NY Acad. Sci. 51, 660–672.
[22] U'Prichard, D. C., Bechtel, W. D., Rouot, B. M. and Snyder, S. H. (1979) Mol. Pharmacol. 16, 47–60.
[23] Wood, C. L., Arnett, C. D., Clarke, W. R., Tsai, B. S. and Lefkowitz, R. J. (1979) Biochem. Pharmacol. 28, 1277–1282.
[24] Schimmel, R. J. (1976) Biochim. Biophys. Acta 428, 379–387.
[25] Williams, L. T. and Lefkowitz, R. J. (1977) Mol. Pharmacol. 13, 304–313.
[26] Guellaën, G. and Hanoune, J. (1979) Biochem. Biophys. Res. Commun. 89, 1178–1185.
[27] Langer, S. Z. (1977) Brit. J. Pharmacol. 60, 481–497.
[28] Giudicelli, Y., Lacasa, D. and Agli, B. (1980) Biochem. Biophys. Res. Commun. in press.
[29] Schimmel, R. J. (1979) Biochim. Biophys. Acta 587, 217–226.
[30] Blackmore, P. F., Assimacopoulos, J., Chan, T. M. and Exton, J. H. (1979) J. Biol. Chem. 254, 2828–2834.