DIRECT BIOCHEMICAL EVIDENCE FOR THE EXISTENCE OF α-ADRENERGIC RECEPTORS IN HAMSTER WHITE ADIPOCYTE MEMBRANES

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1. Introduction

In fat cells, the first evidence confirming the coexistence of functionally opposed α- and β-adrenergic receptors as regulatory subunits of adenylate cyclase [1] was obtained with human adipocytes [2]. However, it was rapidly felt that this concept could not be extended to the fat cells of all species studied and particularly to the commonly used rat fat cells [3].

Recent studies have shown that hamster white fat cells have a peculiar behaviour which set them apart from rat adipocytes [4]. Indeed, experiments testing the influence of catecholamine and phentolamine on cAMP metabolism provided some evidence for the existence of α-adrenergic sensitivity in these cells, suggesting that hamster adipocytes may be an appropriate model to study some aspects of the hormonal control of human fat cells [4]; To date, however, no attempt has been made to directly identify the α-adrenergic receptors in hamster white fat cells.

Recently, methods measuring the binding of the α-adrenergic antagonist [^{3}H]dihydroergocryptine [5] have been successfully used for the direct identification of α-adrenergic receptors in different tissues [6–8] and cells [9]. Using these binding techniques, we now report, in hamster white adipocyte, the identification of [^{3}H]dihydroergocryptine binding sites which have the characteristics expected of α-adrenergic receptors and which appear therefore to represent the physiologically-active hamster adipocyte α-adrenergic receptors.

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2. Material and methods

2.1. *Materials*

[^{3}H]Dihydroergocryptine (spec. act. 22 Ci/mmol) prepared by the Radiochemical Centre (Amersham) and stored in the dark at −20°C in ethanol, had a radiochemical purity of 98% (checked by thin-layer chromatography [7]). Fresh stock solutions, prepared by adding [^{3}H]dihydroergocryptine to an aqueous solution containing 5 mM HCl and 10% ethanol, were diluted 6-fold when added to the binding assay. Under these conditions, ethanol up to 4% in the assay had no effect on the specific binding of [^{3}H]dihydroergocryptine.

(−)-Epinephrine, (−)-norepinephrine, (−)-isoproterenol (all as bitartrate) and ergotamine tartrate were from Sigma. Bitartrate salts of (+)-epinephrine, (+)-norepinephrine and (+)-isoproterenol were gifts from Sterling Winthrop Pharmaceuticals. Phentolamine methane sulfonate and phenoxybenzamine hydrochloride were generously supplied by Ciba-Geigy and Smith, Kline and French, respectively. (−)- and (+)-propranolol hydrochlorides were gifts from ICI-Pharma.

Stock solutions of ergotamine, phentolamine and propranolol were freshly prepared as above for dihydroergocryptine. Stock solutions of phenoxybenzamine prepared at 6×10^{-3} M in absolute

ethanol and diluted in 10 mM $MgCl_2$, 50 mM Tris–HCl, were freshly prepared before use in the binding assays.

2.2. *Preparation of 'crude' adipocyte membranes*

Male golden hamsters (Charles Rivers), 95–105 g, were fed ad libitum before being sacrificed by decapitation. Epididymal fat pads from 15–20 hamsters were pooled and isolated fat cells prepared following the rapid (7 min) collagenase digestion isolation procedure in [10]. Crude adipocyte membranes, prepared as in [11], were finally suspended in 10 mM $MgCl_2$, 50 mM Tris–HCl, resulting in a suspension containing 2–2.5 mg protein/ml which was used in the binding assays. Protein was determined according to [12] using bovine serum albumin as a standard.

2.3. *Binding assays*

Binding assays were performed, using daily-prepared crude membranes according to a slight modification of the procedure in [7]. Protein (300–350 μg) was usually incubated with 15 nM [^{3}H]dihydroergocryptine in total vol. 150 μl incubation buffer (10 mM $MgCl_2$, 50 mM Tris–HCl) for different periods (usually 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 two 7.5 ml portions of buffer. Under these conditions, filtration and washing required less than 30 s. Filters were dried and added to 5 ml scintillation cocktail (Instagel, Packard) and counted in a Kontron MR 300 spectrometer with an efficiency of 40%.

Non-specific binding was determined by measuring the radioactivity retained on filters when incubations were performed in the presence of a large excess (10 or 100 μM) of phentolamine. All values reported refer to specific or receptor binding which is defined as total radioactivity bound minus non-specific binding and which generally averaged 75–85% of the total counts bound.

As attested by chromatographical studies, no degradation of [^{3}H]dihydroergocryptine could be detected during the binding incubations.

2.4. *Other determinations*

The equilibrium dissociation constant, K_d, for the interaction of the binding site with each of the compounds tested for their ability to compete with [^{3}H]dihydroergocryptine was calculated according to [13], from the concentrations of these agents, EC_{50}, that caused 50% inhibition of [^{3}H]dihydroergocryptine binding. Cell numbers were calculated as in [14].

3. Results

3.1. *Number and affinity of binding sites*

The binding of [^{3}H]dihydroergocryptine to hamster white fat cell membranes was a saturable process (fig.1) with 1.1 pmol [^{3}H]dihydroergocryptine bound/mg protein at saturation. Half-maximal saturation occured at ~10 nM [^{3}H]dihydroergocryptine providing an estimate of the K_d for the interaction of [^{3}H]dihydroergocryptine with the binding sites. Scatchard [15] analysis of these binding data suggests

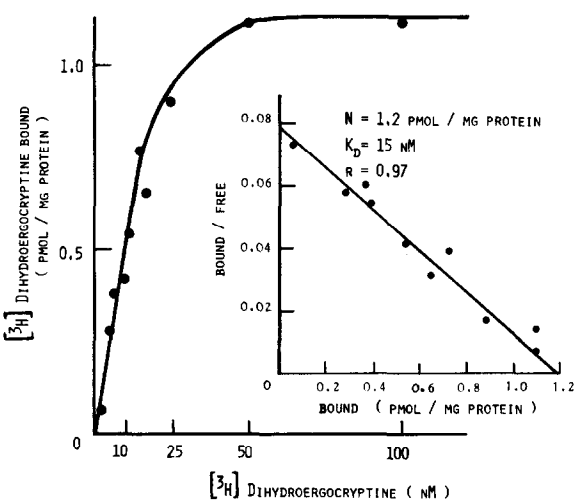

Fig.1. Specific binding of [^{3}H]dihydroergocryptine to hamster adipocyte membranes as a function of [^{3}H]dihydroergocryptine concentration. Hamster adipocyte membranes (2 mg/ml) were incubated with the indicated concentrations of [^{3}H]dihydroergocryptine and specific binding was determined as described under section 2. Each value is the mean of duplicate determinations from two separate experiments. Inset: Scatchard plot of [^{3}H]dihydroergocryptine binding to hamster adipocyte membranes. The ratio (B/F) of bound [^{3}H]dihydroergocryptine to free [^{3}H]dihydroergocryptine is plotted as a function of bound ligand (pM/mg of protein). The slope of the plot, $-1/K_d$, was determined by linear regression analysis (correlation coefficient $r = 0.97$).

a single order of sites with K_d 15 nM (fig.1, inset). Intercept of this plot with the abscissa provides an estimate of the maximal number of binding sites n = 1.2 pmol/mg membrane protein, i.e., a density of 110 000 dihydroergocryptine binding sites per cell.

3.2. *Kinetics of [^{3}H]dihydroergocryptine binding*

The binding of [^{3}H]dihydroergocryptine was rapidly reaching equilibrium within 10 min at 37°C (fig.2) and was reversible (fig.3), dissociation of [^{3}H]dihydroergocryptine from its binding sites following first-order kinetics with a rate constant, k_2, determined by linear regression analysis (r = 0.99), of 0.053 min^{-1} (data not shown).

Since the concentration of dihydroergocryptine (15 nM) was much greater than the binding site concentration in the association assay (1.2 nM), the formation of the dihydroergocryptine–receptor complex could be considered as a pseudo-first order reaction depending only on the binding site concentration. This was evidenced by the linearity of the plot defined by the equation:

$$\ln X_{eq}/(X_{eq}-X) = k_{ob}.t$$

(data not shown) where X_{eq} represents the amount of radioligand bound at equilibrium (0.65 pmol/mg protein) and X is the amount of [^{3}H]dihydroergocryptine bound at each time t; under these conditions, the slope k_{ob} (pseudo-first order rate constant) determined by linear regression analysis (r = 0.99) was equal to 0.30 min^{-1}. The second order rate constant, k_1, computed from $k_1 = (k_{ob} - k_2)$ / DHE, where DHE is the concentration of [^{3}H]dihydroergocryptine in the binding assay, was equal to 1.6 × 10^7 M^{-1} min^{-1}. The value of the ratio k_2/k_1 = 3.2 nM, which provides a kinetically-derived estimate of the K_d of [^{3}H]dihydroergocryptine binding sites, is comparable to the K_d (10–15 nM) derived from equilibrium studies (fig.1.).

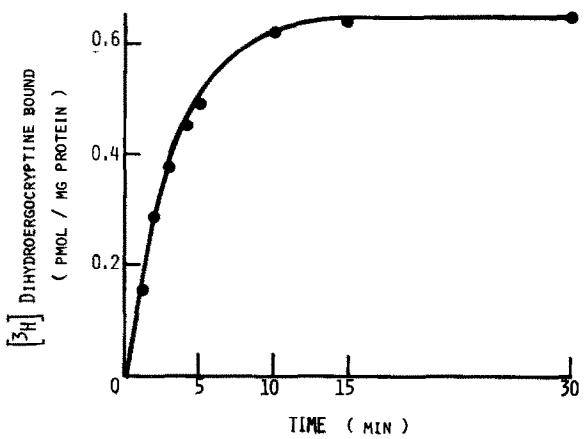

Fig.2. Time course of [^{3}H]dihydroergocryptine binding to hamster adipocyte membranes. [^{3}H]Dihydroergocryptine (15 nM) was incubated with hamster adipocyte membranes for the indicated times at 37°C and specific binding was determined as described under section 2. Each value is the mean of duplicate determinations.

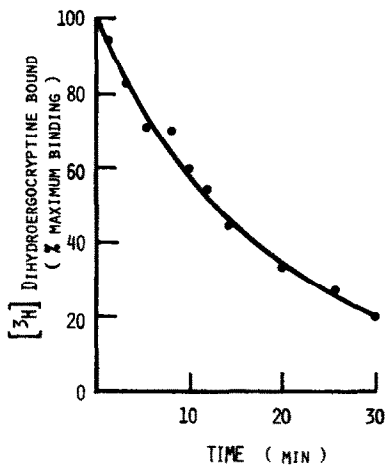

Fig.3. Reversibility of [^{3}H]dihydroergocryptine binding to hamster adipocyte membranes. Membranes were incubated with [^{3}H]dihydroergocryptine (15 nM) at 37°C for 10 min, after which a large excess of phentolamine (100 μM) was added (t = 0). The specific binding of [^{3}H]dihydroergocryptine was determined as a function of time as described under section 2. Each value is the mean of duplicate determinations. Maximum binding is defined as the amount of binding just prior to the addition of phentolamine at t = 0.

3.3. *Specificity of binding*

Adrenergic agonists competed for the [^{3}H]dihydroergocryptine binding sites (fig.4) in the following order of potency:

(–)-epinephrine > (–)-norepinephrine ⩾

(–)-isoproterenol.

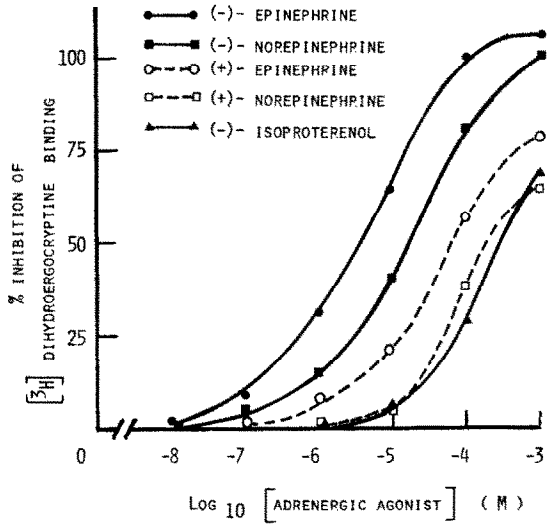

Fig.4. Inhibition of [^{3}H]dihydroergocryptine binding by adrenergic agonists. Hamster adipocyte membranes were incubated with [^{3}H]dihydroergocryptine (15 nM) and with increasing concentrations of the indicated agonists. "100% inhibition" refers to the inhibition of specific binding by 100 μM phentolamine. Each value is the mean of duplicate determinations from two separate experiments.

(−)-Epinephrine and (−)-norepinephrine had high affinity for [^{3}H]dihydroergocryptine binding sites, with respective K_d 2 μM and 8 μM; (−)-isoproterenol, on the contrary, had very low affinity (K_d 150 μM). The [^{3}H]dihydroergocryptine binding sites were highly stereospecific (fig.4), the concentrations of the (+)-stereoisomers of epinephrine and norepinephrine required to half-maximally inhibit the binding being, respectively, 15- and 14-fold higher than those of the corresponding (−)-stereoisomers.

As shown in table 1, the α-adrenergic antagonists phentolamine and phenoxybenzamine potently competed for the binding sites, with respective K_d 50 nM and 90 nM, in good agreement with their reported K_d values as α-adrenergic antagonist in other tissues [16]. In contrast, the β-adrenergic antagonist (−)-propranolol competed for the [^{3}H]dihydroergocryptine binding sites only at very high concentrations (K_d 100 μM).

Finally, dopamine and serotonin were also found to be very weak competitors for the [^{3}H]dihydroergocryptine binding sites (K_d 50 μM and 150 μM, respectively), whereas the potent α-adrenergic ergot alkaloid ergotamine was, among all the compounds tested in this study, the most efficient competitor (K_d 7 nM) (table 1).

Table 1
Inhibition of [^{3}H]dihydroergocryptine binding by different adrenergic and non-adrenergic agents

Compounds	K_d (nM)
(−)-Epinephrine	2000
(−)-Norepinephrine	8000
(+)-Epinephrine	30 000
(+)-Norepinephrine	110 000
(−)-Isoproterenol	150 000
Phentolamine	50
Phenoxybenzamine	90
(−)-Propranolol	100 000
Ergotamine	7
Dopamine	50 000
Serotonine	150 000

The experimental conditions used are those in fig.4. Apparent dissociation constants, K_d, were calculated according to the equation in [13] as in section 2. The K_d values for the (+)- and (−)-stereoisomers of epinephrine and norepinephrine and for (−)-isoproterenol were calculated from the data in fig.4

4. Discussion

Although the existence of α-adrenergic receptors has been postulated in human and hamster adipocytes for over 5 years [2,4], information about the receptors could only be inferred from physiological studies on the ability of various agents to stimulate or block the effects of α-adrenergic agonists on cyclic AMP synthesis or on lipolysis [2,4,10,17,20].

Recently, binding methods using as a ligand either the α-adrenergic antagonist [^{3}H]dihydroergocryptine or the α-adrenergic agonists [^{3}H]clonidine and [^{3}H]norepinephrine with high specific radioactivity have been successfully developed to specifically identify uterine smooth muscle- [6,7], platelets- [9], brain- [8,21] and liver-binding sites [22] which satisfy all the criteria expected of physiological α-adrenergic receptors.

As presently shown, application of these methods to hamster adipocyte membranes has allowed for the first time the identification of [^{3}H]dihydroergo-

cryptine binding sites which have also the characteristics expected of physiological α-adrenergic receptors.

In fact, binding of [^{3}H]dihydroergocryptine was rapid with a time-course compatible with the rapid rate at which α-adrenolytic agents enhance, in vitro, the stimulating effect of norepinephrine on the production of cyclic AMP by intact hamster adipocytes [10]. Binding of [^{3}H]dihydroergocryptine to hamster adipocyte membranes was also saturable, of high affinity and reversible. On the other hand, it was specific, the order of potency with which adrenergic agonists competed for the binding sites being identical with that reported for α-adrenergic receptors in other cells [6–9,21–24] and opposite to the order of potency with which these compounds stimulate both cyclic AMP synthesis and lipolysis in hamster fat cells [17,19]. Moreover, the potent α-adrenolytic drugs, phentolamine and phenoxybenzamine [25], had high affinity for the binding sites, whereas propranolol, a β-adrenolytic agent [25], exhibited a weak inhibition of [^{3}H]dihydroergocryptine binding. Finally, the [^{3}H]dihydroergocryptine binding sites of hamster adipocyte membranes are stereospecific and have a specificity pattern somewhat different from that found in rat brain cortex, in which binding appears to involve, besides the true α-adrenergic receptors, the dopamine and the serotonin receptors as well [26,27]. Experiments using other α-adrenergic antagonists (e.g., prazosin, yohimbine, indoramin) are currently under investigation in order to determine whether the dihydroergocryptine binding sites of hamster fat cells represent one homogenous- or two different α-adrenergic receptor-populations (α_1 and α_2) as recently reported for the rat brain cortex α-receptors [28,29].

The results of this direct binding study provide a more accurate and quantitative assessment of the action of α-adrenolytic agents at the adipocyte α-adrenergic receptor sites than has been provided by the results of experiments such as those investigating the modification of cyclic AMP level [4,10] and lipolysis [10,17]. Furthermore, the present data may provide a tool for future studies on the molecular mechanisms of α-receptor-mediated adrenergic stimulation in adipocyte and especially those concerning the adrenergic control of lipolysis in human fat cells. In fact, while the regulation of the cyclic AMP level via the β-receptor adenylate cyclase system is well described, very little is known about the primary effects linked to the binding of catecholamines to α-receptors. Among these effects is the possible coupling of adipocyte α-receptors to processes regulating the transfer of calcium as recently suggested for the α-adrenergic receptors of other tissues [30,31].

Acknowledgements

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