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DIRECT BINDING STUDIES DO NOT SUPPORT THE EXISTENCE OF TRUE α-ADRENO-RECEPTORS IN RAT WHITE FAT CELLS.

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SUMMARY : By direct binding studies using the mixed α_1-and α_2-antagonist ^{3}H-dihydroergocryptine (^{3}H-DHEC) and the $\alpha_1$antagonist ^{3}H-prazosin as ligands, we have attempted to provide evidence for the existence of α_1-adrenergic receptors in rat adipocyte membranes. Specific binding of both ligands is rapid, is reversible, is of high affinity (K_D for ^{3}H-DHEC and ^{3}H-prazosin : 17.4 ± 2.4 and 12.1 ± 2.7 nM respectively), is saturable (Bmax for ^{3}H-DHEC and ^{3}H-prazosin : 222 ± 15 and 228 ± 37 fmol/mg protein, respectively) and is consistent with binding to a single class of binding sites, suggesting that both ligands label the same sites. Displacement of ^{3}H-DHEC by different adrenergically active drugs showed the following order of potency : prazosin > (-)-isoproterenol > (-)-epinephrine > (+)-epinephrine > yohimbine. These data as well as the inability of (-)-norepinephrine and methoxamine (an α_1-agonist) to displace ^{3}H-DHEC indicate that these ^{3}H-DHEC binding sites are not true α_1-adrenergic receptors and therefore render doubtful the existence of such receptors in rat white adipocytes.

INTRODUCTION :

For a long time, it has been postulated that the adrenergic control of rat adipocyte metabolism was exerted through the β-adrenergic receptors only. Recent reports (1-2), however, raised the possibility that rat fat cells, like the adipocytes of other species (3-6) may be also sensitive to some α-adrenergic-mediated actions of catecholamines, thus suggesting that rat fat cells should also have α-receptors. Physiological studies (2) showed that epinephrine stimulated the turnover of phosphatidylinositol in these cells, an effect which was inhibited by α_1- but not α_2-adrenergic antagonists. In recent studies from our laboratory (7),

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

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functional α_1- and α_2-adrenergic receptors have been identified and characterized in hamster fat cells, using the α-antagonist, (^{3}H)-dihydroergocryptine (^{3}H-DHEC). Applying to rat fat cell membranes the same radioligand binding technique, we now report the presence and some properties of ^{3}H-DHEC binding sites which do not exhibit classical characteristics of α-adrenergic receptors, rendering thus doubtful the existence of such true receptors in rat adipocytes.

EXPERIMENTAL PROCEDURES :

Isolated adipocytes were prepared from epididymal fat pads removed from fed adult Sprague Dawley rats (350-450 g). The fat cells were subsequently disrupted and a crude membrane preparation was obtained as previously described (8). The final pellet was resuspended in 50 mM Tris/HCl, 5 mM $MgCl_2$ buffer (Medium I) at a protein concentration of 2-3 mg/ml, and was subsequently used in the binding assays.

Membranes (200-250 µg) were incubated for 10 min at 37°C with different concentrations (0.5-150 nM) of ^{3}H-DHEC in a total volume of 125 µl of Medium I. Incubations were terminated by diluting the incubation mixture with 5 ml of ice-cold buffer followed by rapid filtration through Whatman GF/C glass fibre filters. Filters were washed rapidly with 15 ml of ice-cold buffer, were dried and were counted in 10 ml PCS scintillation cocktail with an efficiency of 40 %. Specific binding, defined as binding displaceable by 10 µM phentolamine was 50-60 % of total binding at 15-20 nM ^{3}H -DHEC.

In some experiments, binding of ^{3}H-prazosin to adipocyte membranes was also studied. Membranes (200-250 µg) were incubated as above except that the incubations were carried out for 5 min at 25°C with different concentrations of ^{3}H-prazosin in the absence (total binding) or in the presence (non-specific binding) of 10 µM cold prazosin in a total volume of 125 µl. Filtration and washing steps were performed as described above. Specific binding was 75-80 % of total binding at 15-20 nM ^{3}H -prazosin.

Cyclic AMP production was determined as previously described (6) in isolated fat cells after 5 min incubation in the presence of (-)-epinephrine (50 µM) and theophylline (5 mM). Protein was determined according to Lowry et al. (9).

^{3}H -DHEC (spec. act. 22 Ci/mmol) was purchased from the Radiochemical Centre, Amersham. ^{3}H -prazosin (spec. act. 33 Ci/mmol) and cold prazosin were generously provided by Pfizer. (+)-epinephrine (-)-bitartrate was a gift from Sterling Winthrop. Fresh stock solutions of ^{3}H -DHEC, ^{3}H -prazosin, phentolamine (Ciba), methoxamine (Burrough Wellcome), yohimbine, (-)-epinephrine, (-)-norepinephrine and (-)-isoproterenol (Sigma) were prepared as previously described (7,8).

RESULTS AND DISCUSSIONS:

Specific binding of ^{3}H-DHEC was rapid, reaching equilibrium within 5 min at 37°C and was rapidly reversible. Specific ^{3}H -DHEC binding was saturable and was of high affinity (Fig.1,a). Scatchard analysis (Fig. 1,a,inset) revealed a linear plot consistent with the existence of a single class of binding sites. From three different experiments, the calculated number of binding sites at saturation (Bmax) was 222 $\pm$ 15 fmol per mg protein and the dissociation constant K_D was

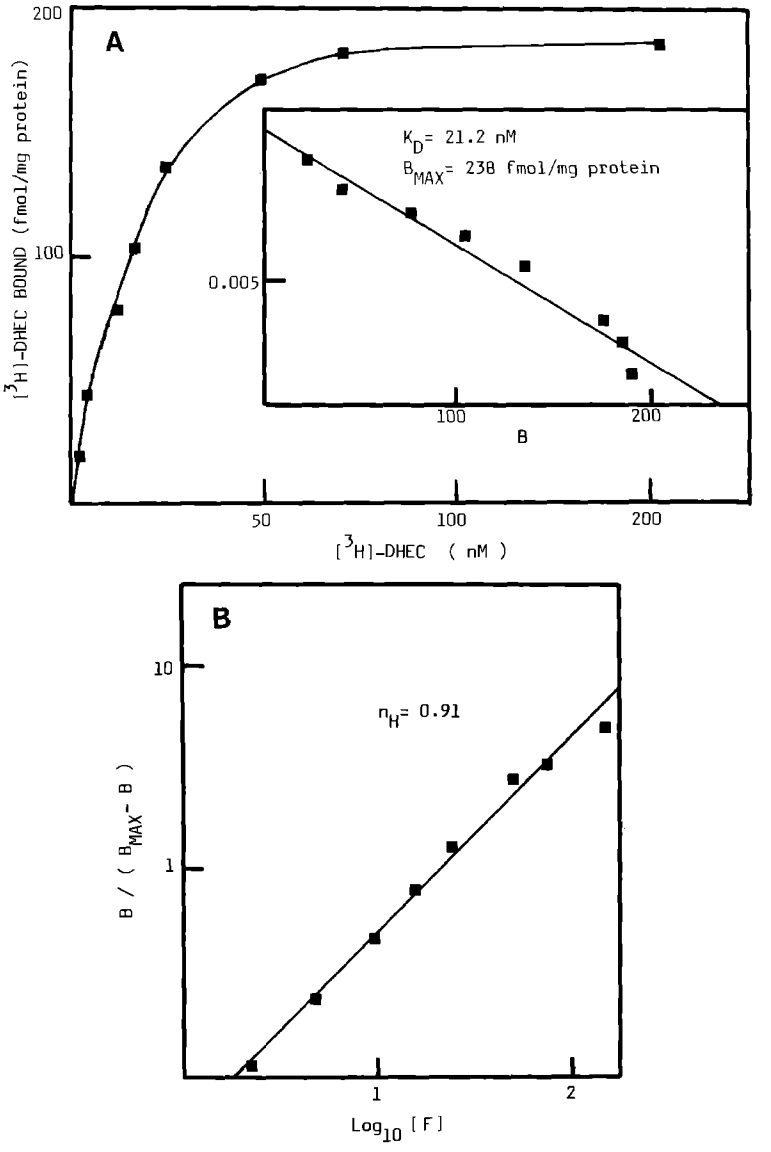

Fig. 1, A. Binding characteristics of ^{3}H-DHEC to rat fat cell membranes. Membranes (200-250 µg) were incubated for 10 min at 37°C with different concentrations of ^{3}H-DHEC (Amersham, 22 Ci per mmol) in a total volume of 125 µl of medium I. Incubations were terminated by diluting the incubation mixture with 5 ml of ice-cold buffer followed by rapid filtration through Whatman GF/c glass fiber filters. Filters were washed with 15 ml of ice-cold buffer, were dried and were counted in 10 ml PCS (Amersham) with an efficiency of 40 %. Specific binding (binding displaceable by 10 µM phentolamine) was 50-60 % of total binding at 15-20 nM ^{3}H-DHEC. B = ^{3}H-DHEC bound to membranes, F = concentration of free ^{3}H-DHEC. Scatchard analysis of these data (inset) yielded a dissociation constant K_D of : 21.2 nM and a maximum number of binding sites of 238 fmol per mg of protein. Fig. 1, B = Hill transformation of ^{3}H-DHEC binding yielded a Hill coefficient of 0.91 (r = 0.98). Each point is the mean of triplicate determination from one representative experiment replicate at least twice.

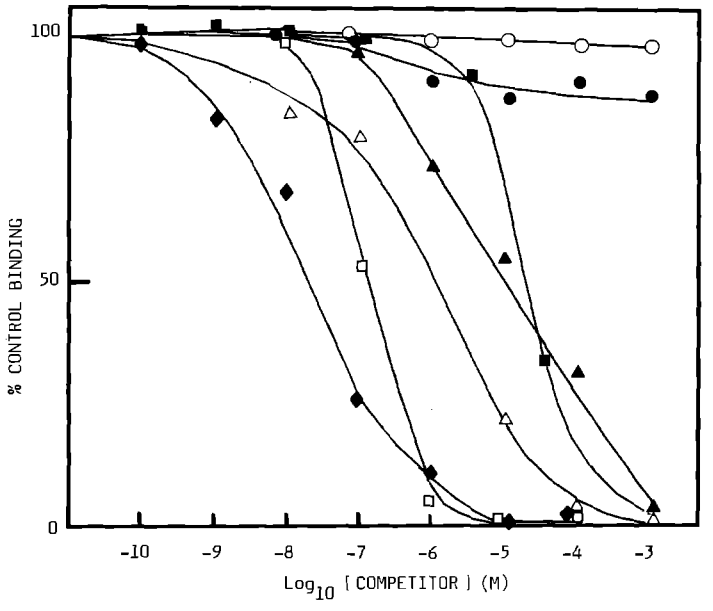

Fig. 2. Displacement curves of ^{3}H-DHEC by different adrenergic drugs. Membranes were incubated with 21 nM of ^{3}H-DHEC for 10 min at 37°C in the absence or presence of increasing concentrations of prazosin (◆), yohimbine (■), (-)-epinephrine (△), (+)-epinephrine (▲), (-)-norepinephrine (●), (-)-isoproterenol (□), methoxamine (○), and the percentage of control specific binding was determined. Each curve is representative of two experiments performed in triplicate.

17.4 ± 2,4 nM (mean ± S.E.M.). Hill plots of these data (Fig. 1,b) were linear with a slope nH = 0.95 ± 0.03, suggesting the absence of cooperative interactions.

In a large number of tissues, ^{3}H-DHEC has been shown to label α_1- and α_2-receptor subtypes with equal affinity(10).To investigate whether these ^{3}H-DHEC specific binding sites of rat fat cells could be pharmacologically classified as α_1- and / or α_2-binding sites, we compared the potency of two selective α_1- and α_2- antagonists, prazosin and yohimbine, respectively, in competing for ^{3}H-DHEC binding. As shown in Fig. 2, yohimbine was a weak competitor (half maximal inhibition of binding, EC_{50} = 15 to 30 μM), whereas prazosin was a potent competitor, yielding apparent EC_{50} value of 25 to 30 nM and a calculated(11)K_D value of 11 to 16 nM. These results provide some pharmacological evidence that these ^{3}H-DHEC binding sites are not, as in hamster adipocytes a mixed population

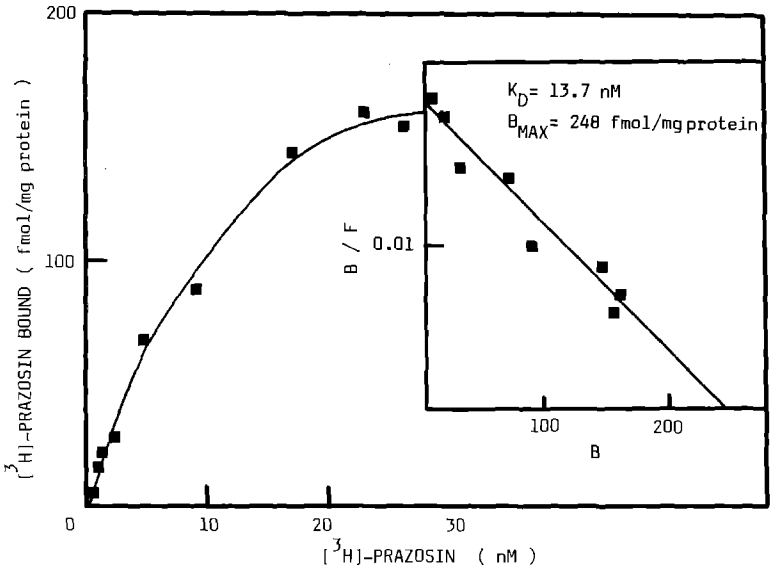

Fig. 3. Binding characteristics of ^{3}H-prazosin to rat adipocyte membranes. Membranes (200-250 µg) were incubated for 5 min at 25°C with different concentrations of ^{3}H-prazosin (Pfizer, 33 Ci per mmol), in the absence (total binding) or in the presence (non-specific binding) of 10 µM prazosin, in a total volume of 125 µl. Filtration and washing steps were performed as described under Fig. 1. Specific binding was 75-80 % of total binding at 15-20 nM ^{3}H-prazosin. Scatchard analysis (inset) of these data yielded a dissociation constant K_D of : 13.7 nM and a maximum number of binding sites of 248 fmol per mg of protein. Each point is the mean of triplicate determinations from one representative experiment replicated at least twice.

of α_1- and α_2-adrenergic binding sites(7), but may be only α_1-antagonist binding sites.

In some tissues, ^{3}H-DHEC has been reported (12) to bind not only to α-receptors but also to other receptors. Therefore, to determine whether the ^{3}H-DHEC binding sites of rat fat cells are exclusively α_1-sites, we compared the binding characteristics of ^{3}H-DHEC with those of ^{3}H-prazosin in the same membrane preparations. Specific binding of ^{3}H-prazosin was optimal at 25°C, was rapid, reaching equilibrium within 5 min, and was very rapidly reversed (complete reversion by the addition of 10 µM prazosin within 1 mn). Specific binding of ^{3}H-prazosin was saturable (Fig. 3) and was of high affinity. Scatchard analysis (Fig. 3, inset) showed a linear plot consistent with the existence of a single class of binding sites. From three different experiments, the calculated Bmax was 228 ± 37 fmol per mg protein,

a value which is equal to the Bmax value found using ^{3}H-DHEC as the ligand and K_D value for prazosin was 12.1 ± 2.7 nM which is also in good agreement with the K_D value derived from the competition experiments depicted in Fig. 2. Hill plots of these data were also linear with slopes nH = 0.97 ± 0.01, suggesting the absence of cooperativity. Thus, these results are consistent with the hypothesis that in rat fat cells, ^{3}H-DHEC and ^{3}H-prazosin label the same sites, which are homogeneous and specific for α_1 but not for α_2- adrenergic antagonists.

To further determine whether these specific binding sites were true α_1 receptors, we studied the potency of different adrenergically active drugs in competing for ^{3}H-DHEC binding sites. As shown in Fig. 2, these sites displayed stereospecificity, since the (-)-isomer of epinephrine was about 10 times more potent than the (+)-isomer ; K_D value for (-)-epinephrine was 1-3 μM, a value in good agreement with the concentration of (-)-epinephrine eliciting half-maximal stimulation of ^{32}P incorporation into phosphatidylinositol in rat adipocytes (2). Surprisingly, (-)-norepinephrine failed to displace bound ^{3}H-DHEC. To explain this unexpected result, additional experiments were performed using both ^{3}H-DHEC and ^{3}H-prazosin as ligands and under different conditions (different ligand concentrations, preincubation of the membranes with (-)-noradrenaline prior to the addition of the ligand, etc...). However, the inability of (-)-norepinephrine to displace ^{3}H-DHEC as well as ^{3}H-prazosin was constantly observed. A more surprising finding was the ability of (-)-isoproterenol (a β-agonist) to displace ^{3}H-DHEC with a potency close to that of prazosin (K_D = 45 nM). Finally, methoxamine, an α_1-agonist failed to compete with ^{3}H-DHEC (Fig. 2). Taken altogether, these competition data led us to conclude that the criteria classically established for characterizing true α-adrenergic receptor sites (13) are clearly not fulfilled by the ^{3}H-DHEC binding sites of rat fat cell membranes.

From the recent literature, there are two reports on the physiological responses of rat adipocytes, claimed to be mediated through α-adrenergic receptors. One is the work of Lawrence and Larner(1) who provide some evidence that the catecholamine-induced inhibition of glycogen synthase and stimulation of phosphorylase,

though mainly regulated by β-activation, could also be mediated through α-adrenergic activation. Moreover, these authors also observed that α-antagonists in the micromolar range potentiated the accumulation of cyclic AMP by norepinephrine, an effect which could not be confirmed by Garcia-Sainz et al.(14) and by us (data not shown). This action is now considered as an α_2-effect in various systems (15), including the hamster(7,14)and the human fat cells(16).The second report is the work of Garcia-Sainz and Fain(2) in which the effect of epinephrine on phosphatidylinositol turnover was mimicked by methoxamine and was suppressed by prazosin, suggesting the α_1-nature of this effect. However, no attempt was made in this study to investigate the effect of epinephrine as well as the stereospecificity of the effect of epinephrine. Moreover, this increase in phosphatidylinositol turnover was also observed with the (+) and the (-) stereoisomers of propranolol, which are unrelated to α_1-receptors.

To conclude, although some physiological studies suggest a possible existence of α-adrenergic receptors in rat fat cells, our attempts to identify and characterize them by direct and conventional binding methods were unsuccessful and render thus their existence doubtful. However, the significance and peculiar properties of the specific ^{3}H-DHEC and ^{3}H-prazosin binding sites reported here are unclear. They may possibly represent the phylogenetic remnants of the true α-adrenoreceptors which are now known to be present in fat cells of various species.

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