# RAT ADIPOCYTE β-ADRENERGIC RECEPTORS: EVIDENCE IN FAVOUR OF THE HETEROGENEITY OF AGONIST-BINDING SITES AND AGAINST NEGATIVELY COOPERATIVE INTERACTIONS

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## 1. Introduction

The direct biochemical identification of β-adrenergic receptors in fat cell membranes was first reported by Williams et al. [1], who showed the existence of specific binding sites for the labeled β-antagonist (−)-[$^3$H]dihydroalprenolol ([$^3$H]DHAP) having the characteristics expected of β-adrenergic receptors. In these studies, transformation of the steady-state [$^3$H]DHAP binding data to a Hill plot [2] yielded an apparent Hill coefficient significantly less than unity (0.65) suggesting the existence of either heterogeneous β-antagonist binding sites with different affinities or negatively cooperative interactions between these binding sites [2]. Confirmation of these peculiar steady-state binding data was later provided by two recent reports, one concerning the influence of altered thyroid states on fat cell adenylate cyclase-coupled β-adrenergic-receptors [3] and the other concerning the influence of age and cell size on adipocyte β-adrenergic receptors [4]. Both of these studies showed, indeed, that Scatchard [5] analysis of the steady-state binding of [$^3$H]DHAP to rat adipocyte membranes yields curvilinear plots with upward concavity.

Since steady-state binding data alone cannot distinguish between the negative cooperativity model and the existence of heterogeneous populations of binding sites, the present investigations were undertaken to determine whether the β-adrenergic receptors of white fat cells display cooperative site–site interactions or not. During the course of these investigations, a report [6] provided some evidence against the existence of negatively cooperative interactions among the β-adrenergic antagonist-binding sites of rat fat cell membranes. In the present studies, we not only confirm this interpretation but also provide some evidence in favour of the heterogeneity of adipocyte β-adrenergic agonist binding sites.

*Abbreviations:* [$^3$H]DHAP, (−)-[$^3$H]dihydroalprenolol; Gpp(NH)p, guanosine 5′-(β,γ-imino)triphosphate

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## 2. Materials and methods

### 2.1. *Preparation of crude adipocyte membranes*

Male Wistar rats (CERJ, 225–250 g) were fed ad libitum before being sacrificed by decapitation. Epididymal fat pads from 10–15 rats were pooled and isolated fat cells prepared as in [7]. Crude adipocyte membranes prepared as in [4], were finally suspended in 10 mM $MgCl_2$, 50 mM Tris–HCl (medium I) resulting in a suspension containing 3–4 mg protein/ml which was used in the binding assays. Protein was determined according to Lowry [8] using bovine serum albumin as a standard.

### 2.2. *Binding assays*

Binding assays were performed using daily-prepared crude membranes as in [4,9]. In brief, membrane (150–175 μg) was incubated usually with 20 nM

[$^3$H]DHAP in medium I for 8 min with shaking at 37°C. Incubations were terminated by adding ice-cold buffer followed by rapid vacuum filtration through a Whatman GFC glass fiber filter. Filters were rapidly washed, dried, added to scintillation cocktail (MI 96, Packard) and counted. Non-specific binding, determined by measuring the radioactivity remaining on filters when incubations were performed with a large excess (20 μM) of (±)-alprenolol, averaged 20–25% of the counts specifically bound to adipocyte membranes.

### 2.3. *Dissociation kinetics studies*

Negative cooperativity among β-receptors was assessed by applying the direct kinetic method [10]. Membranes were incubated with 15 nM [$^3$H]DHAP in medium I at 25°C for 30 min (time corresponding to binding equilibrium at this temperature), after which 100 μl aliquots were transferred in 9.9 ml medium I containing or not 10 μM unlabeled (−)-propranolol, (−)-alprenolol or (−)-isoproterenol and further incubated at 25 or 37°C. At appropriate intervals, duplicates from each set (with or without unlabeled compounds) were filtered, washed, dried and counted as described above. Radioactivity specifically bound to the membranes was expressed as percentage of the radioactivity specifically bound at time zero (time immediately after the 1:100 dilutions).

### 2.4. *Other determinations*

In competition experiments, equilibrium dissociation constant, $K_d$, for the interaction of the binding sites with each of the unlabeled compounds tested for their ability to compete with [$^3$H]DHAP was calculated as in [11]. In these experiments, analysis of the resulting data by Hill plots was performed taking as $B_{max}$, the specific radioligand binding occurring in the absence of any displacing agent, and as $B$ the percentage of $B_{max}$ displaced at any concentration of displacing agents; data were then plotted as log (% $B_{max}$/100%−% $B_{max}$) versus log (displacer) to yield the Hill plot [12].

### 2.5. *Chemicals*

(−)-[$^3$H]Dihydroalprenolol (spec. act. 39 Ci/mmol) was prepared by the Radiochemical Centre (Amersham) with a radiochemical purity (monitored by thin-layer chromatography) >98%. (±)-Alprenolol was a gift from Laboratories Lematte et Boinot. Other compounds used in this study were: (−)-isoproterenol bitartrate (Sigma Chemical Co., USA), (−)-propranolol which was kindly supplied by ICI Pharma (France) and 5′-guanylyl-imidodiphosphate (Gpp(NH)p) from Boehringer (FRG).

## 3. Results

Hill transformation of the steady-state specific binding of the β-antagonist [$^3$H]DHAP to adipocyte membranes yielded an apparent Hill coefficient of 0.73 with a dissociation constant $K_d$ of 22 nM (data not shown), confirming thus published results [1,3,4].

The specific binding of β-adrenergic agonists to adipocyte membranes was studied by measuring the

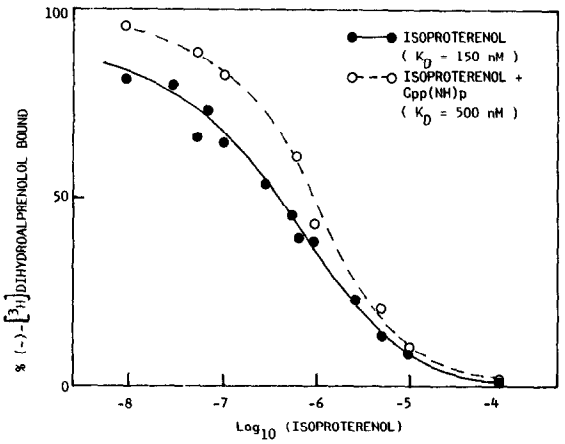


Fig.1. (−)-Isoproterenol binding to the β-adrenergic receptors of rat fat cell membranes. Effect of Gpp(NH)p. Specific binding of (−)-isoproterenol in the absence or presence of 100 μM Gpp(NH)p was assayed by competition for [$^3$H]dihydroalprenolol binding sites as follows: membranes were incubated with 20 nM [$^3$H]dihydroalprenolol and with increasing concentrations of (−)-isoproterenol in the absence or presence of 100 μM Gpp(NH)p. The amount of dihydroalprenolol specifically bound was then determined and the results expressed as percent of the amount bound in the absence of (−)-isoproterenol. Data concerning (−)-isoproterenol alone are means of 4 separate experiments performed in triplicate. Data concerning Gpp(NH)p + (−)-isoproterenol are means of two experiments performed in triplicate. Dissociation constants calculated according to [11], were: $K_d$ for isoproterenol = 150 nM; $K_d$ for isoproterenol with Gpp(NH)p = 500 nM.

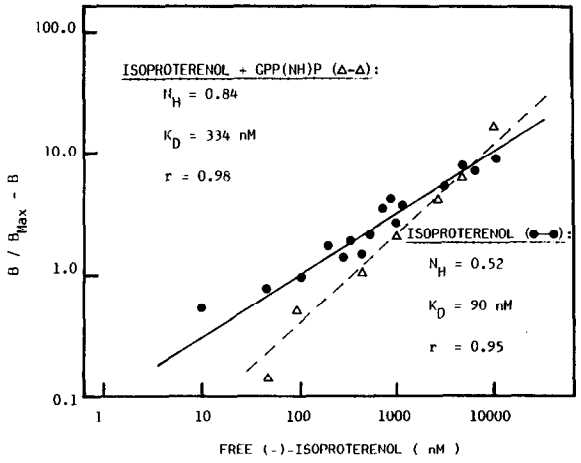


Fig.2. Apparent Hill coefficient of (−)-isoproterenol binding to adipocyte β-adrenergic receptors. Effect of Gpp(NH)p. Data in fig.1 were transformed into Hill plots as in section 2. Apparent Hill coefficient $n_H$ and dissociation constants $K_d$ values given in the figure were calculated by regression analysis.

displacement of [$^3$H]DHAP specifically bound by increased (−)-isoproterenol concentrations. As shown in fig.1, the resulting competition curve was rather broad yielded an app. $K_d$ for (−)-isoproterenol of 0.15 ± 0.09 μM (mean ± SEM of 4 separate experiments). These competition data have been transformed as Hill plots as in section 2 (fig.2) and as Scatchard plots (fig.3) following a modified Scatchard analysis [5] in which the amount of specific [$^3$H]-DHAP binding displaced at each (−)-isoproterenol concentration has been assumed to represent the amount of agonist specifically bound at the corresponding agonist concentration.

This type of analysis was acceptable under the experimental conditions used since the fraction of total [$^3$H]DHAP bound to the membranes in the absence of competition ligand was <10% of the free radioactive or competitive ligand, indicating that the concentration of these ligands is relatively constant and that variations in these fractions would be unlikely to influence graphical analysis. As shown in fig.2, the Hill plot drawn from the data in fig.1, yielded an apparent Hill number of 0.52 ± 0.08 (mean ± SEM of 4 separate experiments), a value which is consistant with either negatively cooperative interactions between agonist receptor sites or with the existence of two or more independent binding sites. Scatchard analysis of the data in fig.1 yielded, as in the case of β-adrenergic antagonist binding [2–4], a peculiar curvilinear plot with upward concavity (fig.4). Separate regression analysis of the data obtained with isoproterenol concentrations at ⩽500 nM and of the data obtained with higher concentrations provided a strong evidence in favour of the existence of two apparent orders of binding sites, one with high affinity for (−)-isoproterenol ($K_d$ = 33 nM, $r$ = 0.95) and one with low affinity ($K_d$ = 700 nM, $r$ = 0.95).

As shown in fig.1, the slope of the competition curve for (−)-isoproterenol approached that described by a Hill coefficient of 1, when competition was performed in the presence of 100 μM Gpp(NH)p, a nucleotide which reduces the β-receptor affinity for agonists in adipocytes [13] as it does in other cells [14,15]. This was confirmed by the data in fig.2 showing that Gpp(NH)p induces indeed a shift of the apparent Hill

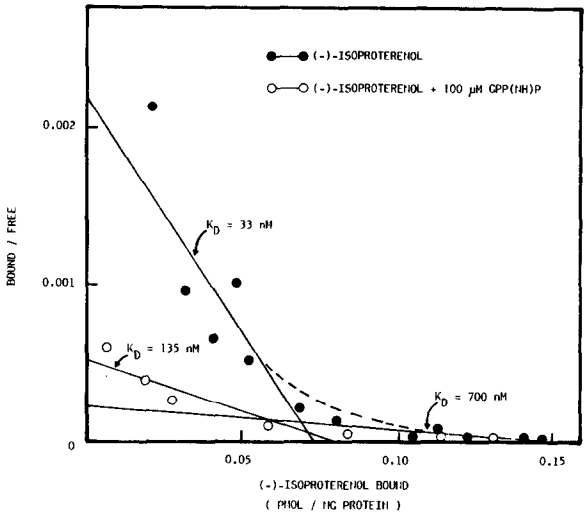


Fig.3. Scatchard analysis of (−)-isoproterenol binding to the β-adrenergic receptors of rat fat cell membranes. Effect of Gpp(NH)p. Binding data of fig.1 were analysed according to Scatchard [5]. The apparent two orders of binding sites and their respective dissociation constants were calculated by regression analysis. The point of inflection between the lines was arbitrarily decided when the high affinity component giving the highest correlation coefficient was calculated. $K_d$ for the high affinity sites (isoproterenol concentrations between 10 and 500 nM) was 33 nM with a correlation coefficient $r$ = 0.95; for the low affinity sites (isoproterenol concentrations above 500 nM), the calculated $K_d$ value was 700 nM with $r$ = 0.95.

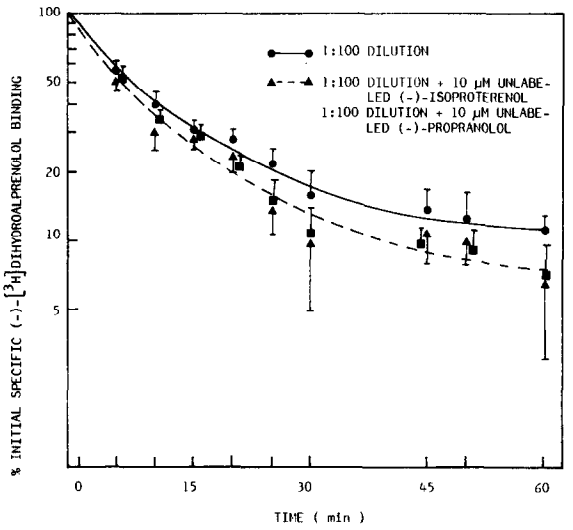


Fig.4. Ability of β-adrenergic agonists and antagonists to increase the rate of dissociation of specific [$^3$H]dihydroalprenolol binding. Membranes were preincubated with 15 nM [$^3$H]dihydroalprenolol at 25°C for 30 min, then diluted 1:100-fold into buffer containing or not the unlabeled β-adrenergic agonist or antagonist and further incubated at 25°C. Samples were filtered at the indicated times and counted for specific binding. Each point is the mean of 2 separate experiments performed in duplicate (vertical lines = 1 SEM).

number of (−)-isoproterenol binding to a higher value ($n_H = 0.84, r = 0.98$) which could be more consistant with the existence of a single set of agonist binding sites, under these conditions. On the other hand, Scatchard analysis of (−)-isoproterenol binding in the presence of Gpp(NH)p, indicates that this nucleotide selectively reduces the affinity of the apparent high affinity order of binding sites (fig.3).

Existence of negatively cooperative site–site interaction was tested by studying the influence of β-adrenergic ligands on the kinetics of the [$^3$H]DHAP–receptor complex dissociation that follows a 1 : 100 dilution of membranes containing an excess of free receptors. These conditions were found to be adequate to prevent [$^3$H]DHAP rebinding during the dissociation phase.

As shown in fig.4, addition of an excess (10 μM) of (−)-isoproterenol or (−)-propranolol resulted in an only slight (4–9%) and non-significant increase in the [$^3$H]DHAP dissociation rate at 25°C as compared with dissociation by dilution alone.

## 4. Discussion

Several reports have shown that the steady-state binding of radioligands to some hormone-receptors yields Hill numbers of <1.0 and/or curvilinear Scatchard plots with upward concavity. For some of these hormones [10,16–21], existence of negative cooperativity among their specific receptors has been postulated to be the basis of this non-classical way of binding. Indeed, the dissociation of the labeled hormones from their specific receptors was demonstrated to be significantly accelerated by unlabeled hormones in the case of polypeptide hormones such as insulin [10,16] and nerve growth factor [17,18] and glycopeptide hormone such as thyroid stimulating hormone [19]. Thus, negative cooperativity among hormones and their receptors appears to be a rather widespread phenomenon.

In the case of catecholamines, however, negative cooperativity among β-adrenergic receptors seems so far to be specific to the frog erythrocyte [2] and Hela cell [21] membranes. In fact, numerous recent studies on the β-adrenergic receptors of turkey erythrocytes [22], rat liver [23], brain [24,25], glioma cells [26], parotid [27], erythrocytes [28], skeletal muscle [29] and heart [30], strongly suggest the existence of one type of binding site displaying no apparent negatively cooperative site–site interactions. Moreover, evidence against negative cooperativity among the β-adrenergic receptors of another catecholamine target cell, the adipocyte was recently provided by Malbon and Cabelli [6] who reported an insignificant acceleration of [$^3$H]DHAP dissociation by antagonists and agonists, a result which is confirmed here.

Analysis of the steady-state binding of [$^3$H]DHAP and of (−)-isoproterenol (drawn from competition curves) to rat fat liver membranes yields Hill plots with Hill numbers of <1.0 (0.73 for [$^3$H]DHAP and 0.52 for (−)-isoproterenol). Thus, because of the absence of discernable negative cooperativity, the most likely explanation for these low $n_H$ values is that β-adrenergic antagonist- and agonist-binding sites exist in the fat cell as multiple populations with different affinities.

In the case of (−)-isoproterenol-receptor sites, the data reported in fig.3 are in favour of the existence, in approximately equivalent proportions, of two apparent orders of binding sites with, respectively,

high and low affinity for (−)-isoproterenol. This apparent dual binding, a phenomenon which has been already found in our laboratory for the specific but non-receptor (−)-[$^3$H]norepinephrine binding sites of intact rat epididymal fat cells [31], could reflect the heterogeneous nature of the biological preparation used (crude membranes). This hypothesis appears, however, unlikely if one considers the data [1] showing that the specificity and affinity characteristics of the [$^3$H]DHAP-receptor sites of a highly enriched plasma membrane fraction of rat adipocytes are virtually identical with those of the corresponding unfractionated membranes.

The existence of such two orders of binding sites for (−)-isoproterenol in adipocyte membranes is to be compared with the results of [32]. These authors, using also the Scatchard analysis of [$^3$H]DHAP binding inhibition by selective $\beta_1$ and $\beta_2$ competitive ligands (agonists and antagonists, showed indeed the existence of two distinct orders of binding sites for each $\beta_1$- and $\beta_2$-adrenergic receptor subtypes of rat and rabbit lung membranes [32]. In the case of rat fat cells, $\beta$-adrenergic responses are predominantly if not exclusively of the $\beta_1$-subtype [33]. It is thus tempting to consider that the 2 apparent (−)-isoproterenol binding sites presently found represent 2 distinct isoproterenol binding sites among which only the high affinity one, because of its low app. $K_d$, which is more compatible with the nM range of blood catecholamine levels [34] than the $K_d$ of the low affinity binding site, may well represent the true active $\beta_1$-adrenergic receptors of rat fat cells. As an additional support to this hypothesis is the fact that the affinity of these binding sites appears to be selectively altered by Gpp(NH)p, an analog of GTP which is involved in the coupling of adenylate cyclase to the $\beta$-adrenergic receptors [35]. Finally, if these apparently high affinity binding sites represent the true $\beta$-adrenergic receptors of rat fat cells, this would indicate that methods based on the measurements of [$^3$H]DHAP binding may overestimate and consequently be inadequate to assess the number of $\beta$-adrenergic receptors in these preparations. Experiments using the radiolabeled $\beta$-agonist (−)-[$^3$H]-hydroxybenzylisoproterenol [36] are currently in progress to test the validity of these hypotheses.

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