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NON-ADRENERGIC SITES FOR IMIDAZOLINES ARE NOT DIRECTLY INVOLVED IN THE α_2-ADRENERGIC ANTILIPOLYTIC EFFECT OF UK 14304 IN RAT ADIPOCYTES

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Abstract—The binding of the α_2-agonist [^{3}H]UK 14304 on Wistar rat adipocyte membranes was separated in two distinct components: one was displaceable by adrenaline or other α_2-adrenergic agents and possessed the characteristics of α_2-adrenoceptors while the other, non-adrenergic in nature, was only recognized by some imidazoline derivatives. [^{3}H]Idazoxan binding shared the same characteristics. The non-adrenergic sites labeled by both radioligands are similar to those described for [^{3}H]idazoxan on other tissues such as brain cortex, smooth muscle and kidney. Even though they were about 10-fold more numerous than the true α_2-adrenoceptors, the non-adrenergic binding sites were not directly involved in the antilipolytic action of UK 14304 since α_2-antagonists devoid of interaction with these sites (yohimbine, phentolamine) totally blocked the UK 14304 effect. However, the existence of such a type of site impairs direct quantification of α_2-adrenoceptors in rat adipocytes. The use of [^{3}H]RX 821002 (2-(2-methoxy-1,4-benzodioxan-2yl)imidazoline) allowed an accurate quantification of rat adipocyte α_2-adrenoceptors ($B_{max} = 35 \pm 2$ fmol/mg protein, $K_d = 2.6 \pm 0.6$ nM) since it did not interact with non-adrenergic binding sites and exhibited the highest α_2-blocking properties among the various α_2-antagonists tested. [^{3}H]RX 821002 binding analysis revealed that α_2-adrenoceptors are, on rat adipocytes; (i) less numerous than in other species well known for their α_2-adrenergic inhibitory regulation of lipolysis (human, hamster, rabbit); (ii) slightly different in nature from the receptors of these species since they had weaker affinity for clonidine and yohimbine; and however (iii) not of the typical α_2-B subtype since the affinity of prazosin was lower than that of oxymetazoline in displacing [^{3}H]RX 821002 or [^{3}H]yohimbine binding.

We recently described the presence of functional α_2-adrenoceptors on rat white adipocytes [1] using the α_2-agonist UK 14304 [2] which induced a significant antilipolytic response and labeled a high number of receptors (750 fmol/mg plasma membrane protein). This binding capacity was, however, strikingly higher than that commonly described in the adipocytes of various species including human [3], hamster [4] and dog [5]. In fact, the presence of an α-adrenergic receptivity long remained controversial in rat adipocytes since typical α-adrenergic effects [6] but not receptors [7] were reported. In addition, we showed that clonidine (a partial α_2-agonist) was devoid of antilipolytic action and binding capacities in this species [8]. So, it seems unlikely from the [^{3}H]UK 14304 binding data, that on rat adipocytes, α_2-adrenoceptor number is higher than in other species well known to exhibit a good antilipolytic α_2-adrenergic response. In order to explain this discrepancy we made a fuller characterization of the antilipolytic response and of the binding characteristics of UK 14304 on rat white fat cells. Since we used the potent α_2-antagonist idazoxan [9] for the determination of non-specific [^{3}H]UK 14304 binding, our attention was focused on the fact that idazoxan can identify, in addition to α_2-adrenergic receptors, another kind of binding site (non-adrenergic in nature) in several cell models [10–13] including the rabbit adipocyte [14].

The re-assessment of [^{3}H]UK 14304 binding on rat adipocytes reported below provides evidence that this α_2-agonist, like the α_2-antagonist idazoxan, not only binds to α_2-adrenoceptors but also to non-adrenergic sites exhibiting a high affinity for imidazoline derivatives. However, these non-adrenergic sites did not appear to be directly involved in the UK 14304-induced antilipolysis. It is also shown that RX 821002, an idazoxan derivative [15], which does not recognize these sites and exhibits high α_2-adrenergic blocking capacities, is the most suitable ligand for α_2-site identification.

MATERIALS AND METHODS

Animals and fat-cell preparation. Since previous work showed that α_2-responsiveness is developed only in mature animals [1], all the studies were performed on adult Wistar rats weighing between 350 and 420 g housed at 20–22° and fed *ad lib*. The animals were killed by decapitation after overnight fasting. White adipose tissue from different fat deposits (perirenal, epididymal) was immediately removed and pooled. Adipocytes were isolated by the method of Rodbell [16] with minor modifications.

For studies of lipolysis, isolated adipocytes (20–30 mg of total cell lipids) were dispersed in 1 mL of Krebs–Ringer bicarbonate buffer containing 10 mM

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Hepes, pH 7.4, 6 mM glucose and 35 mg/mL of bovine serum albumin. Freshly prepared drugs were added in a volume of 10 μL to plastic vials just before a 90-min incubation at 37° under constant shaking. The reaction was stopped in ice and an aliquot (200 μL) of the incubation medium was taken to determine glycerol by the method of Wieland [17]. The α_2-adrenergic antilipolytic effects were explored on adenosine deaminase-stimulated lipolysis (3 μg/mL) in order to inactivate endogenous adenosine released during incubation which could exert an antilipolytic action. Since adenosine deaminase stimulation was greater in cells incubated under air atmosphere than under O_2/CO_2 (19:1) (2.33 ± 0.31 vs 0.97 ± 0.10 μmol glycerol/10^6 cells/90 min, N = 4, $P < 0.02$), incubations were carried out in non-stoppered plastic vials.

Binding experiments. Crude membranes were obtained and incubations and filtrations were carried out as previously described for hamster adipocytes [18]. All incubations were at 25° in a final volume of 400 μL and lasted 30 min since steady state was reached at this moment for all the radioligands used. The following incubation medium: 50 mM Tris–HCl, 10 mM $MgCl_2$ buffer, pH 7.5, was used for all the radioligands except for [^{3}H]RX 821002 where the $MgCl_2$ concentration was 0.5 mM. For saturation and competition studies, binding parameters (B_{max}, K_d, n_H, IC_{50}) were determined by computer-assisted linear transformation of the data [19].

In the competition experiments, results were expressed as a percentage of the total radioligand binding in order to objectivate all the components of binding: displaceable by adrenaline, displaceable by various drugs and even non-displaceable at all or due to irrelevant material such as the glass fiber filters themselves. This last component of binding did not exceed 5% of total binding for [^{3}H]UK 14304, [^{3}H]idazoxan or 10% for [^{3}H]RX 821002. All the competitors used were freely soluble in incubation medium except for yohimbine and prazosin at the higher doses used (10^{-4} M): they were dissolved in ethanol at a final concentration of 0.25% leaving ligand binding unaltered. The protein content of the membrane preparations was measured by the method of Lowry *et al.* [20] using bovine serum albumin as standard. Final concentrations of membrane protein ranged from 0.35 to 0.75 mg/mL incubation medium.

Drugs. [^{3}H]UK 14304 (3 TBq/mmol) and [^{3}H]clonidine (1.8 TBq/mmol) were purchased from N.E.N. (Boston, MA); [^{3}H]idazoxan (1.7 TBq/mmol), and [^{3}H]yohimbine (3.3 TBq/mmol) were obtained from Amersham France (Les Ulis, France). RX 821002 (2-(2-methoxy-1,4-benzodioxan-2yl)-2 imidazoline), idazoxan (RX 781094) and [^{3}H]RX 821002 (1.6 TBq/mmol) were kindly given by Reckitt & Colman (Hull, U.K.). UK 14304 and prazosin were obtained from Pfizer (Sandwich, U.K.). Clonidine HCl and BHT 920 were kindly given by Boehringer Ingelheim (Reims, France). Phentolamine mesylate was obtained from Ciba Geigy (Basel, Switzerland); (+) and (−)adrenaline from Winthrop (New York). Tramazoline and guanfacine were generous gifts from Dr Huchet-Brisac A.M. (INSERM U.228, Paris, France). Albumin and all other drugs came from the Sigma Chemical Co. (St Louis, MO). Enzymes came from Boehringer Mannheim (Mannheim, F.R.G.).

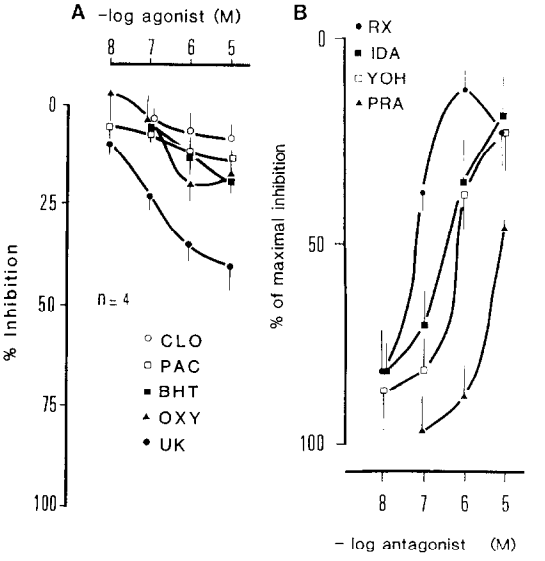

Fig. 1. Pharmacological study of the α_2-adrenergic antilipolytic response. (A) Antilipolytic responses of rat adipocytes to various α_2-agonists are expressed in % inhibition of adenosine deaminase (ADA) stimulated lipolysis. 0% corresponds to 5 μg/mL ADA alone and 100% to basal values (1.13 ± 0.09 and 0.26 ± 0.03 μmoles glycerol/100 mg lipid/90 min, N = 4). Release of glycerol was measured in the presence of increasing concentrations of: CLO: clonidine, PAC: *para*-amino-clonidine, BHT: BHT 920, OXY: oxymetazoline, UK: UK 14304. (B) Action of α-antagonists on UK 14304-induced antilipolysis is expressed in % of the maximal inhibition of UK 14304 (1 μM) on ADA-stimulated (3 μg/mL) lipolysis. Increasing concentrations of RX: RX 821002, IDA: idazoxan, YOH: yohimbine, PRA: prazosin were tested in the presence of ADA + UK 14304. Each point represents mean ± SE of 5–7 experiments.

Statistics. Data are expressed as means ± SE and were analysed by Student's *t*-test; N refers to the number of experiments.

RESULTS AND DISCUSSION

Antilipolytic action of UK 14304

To verify whether the antilipolytic action of UK 14304 is an α_2-adrenergic mechanism, the effects of UK 14304 on rat adipocytes were firstly compared to the effects of other α_2-adrenergic agonists and, secondly, tested in the presence of α-antagonists. Since antilipolytic responses can only be clearly evidenced in adipocytes exhibiting enhanced lipolytic rates, the effects of UK 14304 were measured on adenosine deaminase-stimulated lipolysis. This approach is a valuable method for the investigation of the α_2-adrenergic receptivity in human [21] and hamster fat cells [18]. In rat fat cells, UK 14304 was the most efficient among the α_2-agonists tested (Fig. 1A). At the highest dose used (10^{-5} M), UK 14304 induced 40 ± 6% inhibition of adenosine deaminase-stimulated lipolysis whereas the responses to the

other α_2-agonists did not exceed 25%. A similar observation was also made when lipolysis was stimulated by 1 mM theophylline: UK 14304-induced antilipolysis (48 ± 4% inhibition, N = 4) was stronger than that of oxymetazoline, *para*-aminoclonidine, BHT 920, clonidine, tramazoline, xylazine or guanfacine (not shown). Nevertheless, the high antilipolytic effect of UK 14304 could be linked to its greater intrinsic activity as an α_2-agonist or to a possible "extra-adrenergic" antilipolytic action of the drug or both. The fact that 1 μM UK 14304 was able to inhibit the stimulation of lipolysis induced by forskolin (27 ± 5% inhibition with 1 μM forskolin, N = 4) but not by dibutyryl cAMP (2.18 ± 0.13 vs 2.12 ± 0.13 μmol/100 mg lipid/90 min for dibutyryl cAMP 1 mM alone and in the presence of UK 14304 respectively, N = 4, not significant) indicated that a lowering of cAMP levels is required for the expression of the antilipolytic response of UK 14304. In addition, the antilipolytic action of UK 14304 was blocked in a dose-dependent manner by the α_2-antagonists: yohimbine, idazoxan and RX 821002 (Fig. 1b). The mixed α_1- α_2-antagonist phentolamine was also able to reverse the UK 14304 effect (43 ± 11% inhibition of lipolysis for 1 μM UK 14304 alone vs 14 ± 3% in the presence of 1 μM phentolamine, N = 4, $P < 0.05$). On the contrary, prazosin, an α_1-antagonist was ineffective except at 10^{-5} M where it partially inhibited the UK 14304 effect (Fig. 1B). None of the α_2-antagonists mentioned above blocked the antilipolytic responses promoted by adenosine, PGE_2 or nicotinic acid (not shown), thus their blocking properties against UK 14304 indicated that the antilipolytic action of this drug is, in rat adipocytes like in other species [4, 5, 21], an α_2-adrenergic mechanism.

However, when measured in similar conditions, the maximal antilipolytic response initiated by UK 14304 was reduced in rat fat cells (≈ 40% inhibition of lipolysis) as compared to that of other species (70–90% inhibition in hamster and human) [18, 21]. Such interspecific differences were not found for the antilipolytic responses involving PGE_2 or adenosine receptors (not shown). So, to verify whether this difference in α_2-antilipolytic responsiveness was due to a difference in the α_2-adrenoceptor equipment of the fat cells, binding experiments were carried out on rat adipocyte membranes. Moreover, attention was also focused on the determination of the pharmacological nature of the sites labeled by [^{3}H]UK 14304.

Binding of [^{3}H]*UK* 14304 *and* [^{3}H]*idazoxan to rat adipocyte membranes*

Kinetic studies were performed to assess optimal binding conditions: steady state was reached after 20 min for both radioligands. Saturation curves of [^{3}H]UK 14304 binding to crude membranes of rat adipocytes are shown in Fig. 2. As previously reported [1], 10^{-5} M idazoxan displaced about half the total [^{3}H]UK 14304 binding. Residual binding determined in the presence of this α_2-antagonist was linear with increasing concentrations of [^{3}H]UK 14304. Subtracting the values of this "non-specific" binding from the values of total [^{3}H]UK 14304 binding gave saturable binding characterized by: B_{max} = 120 ± 10 fmol/mg protein, K_d = 5.5 ± 0.6 nM (N = 5). Scatchard plots were linear and the Hill coefficient was not different from unity, reflecting the presence of only one class of binding sites as previously reported in Percoll-purified plasma membranes [1].

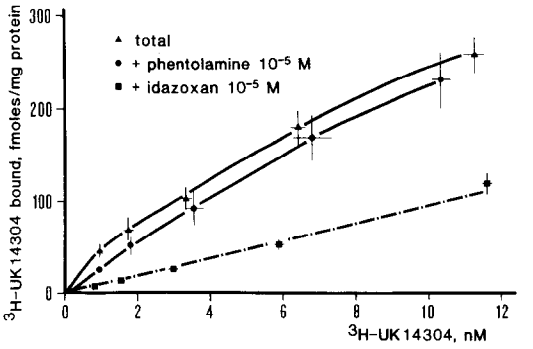

Fig. 2. [^{3}H]UK 14304 binding to crude membranes of rat adipocyte. Langmuir isotherms are shown for total binding (▲) (increasing concentrations of [^{3}H]UK 14304 alone) and non-specific binding in the presence of 10^{-5} M phentolamine (●) or 10^{-5} M idazoxan (■). Binding was determined as described in Materials and Methods. Data are means ± SE of five different experiments.

However, inhibition of total [^{3}H]UK 14304 binding by another α-antagonist—phentolamine which is commonly used for the definition of non-specific binding of α_2-adrenergic radioligands [10, 18]—did not show a similar pattern (Fig. 2). Phentolamine only inhibited 10% of the total [^{3}H]UK 14304 binding and the resulting "specific binding" was unexpectedly very different from that defined with idazoxan in the same conditions: B_{max} = 16 ± 5 fmol/mg protein, K_d = 3.3 ± 0.5 nM, N = 4. The discrepancies between the maximal competing capacities of the two α-antagonists on [^{3}H]UK 14304 binding raised the question of the true nature of the sites identified with this radioligand.

To determine whether the difference between the phentolamine and idazoxan definition of "non-specific" binding values was also obtained with other α-adrenergic agents, competition of total [^{3}H]UK 14304 binding was measured in the presence of high concentrations (10^{-4} M) of various competitors. Strikingly, the maximal displacing capacities of the various competitors used were very different from one another (Fig. 3A). Like idazoxan, the α_2-agonists UK 14304, tramazoline, guanfacine and oxymetazoline fully competed for [^{3}H]UK 14304 binding whereas the other α_2-adrenergic agents such as yohimbine, phentolamine, RX 821002 (α_2-antagonists) and clonidine, BHT 920, xylazine (α_2-agonists) only prevented 30% of [^{3}H]UK 14304 binding. Since adrenaline, the physiological agonist, competed for only 30% of [^{3}H]UK 14304 binding, the adrenergic nature of most of the [^{3}H]UK 14304 binding sites is thus questionable.

Non-adrenergic [^{3}H]idazoxan binding sites have recently been described in the kidney of various species such as rabbit [10], pig [12], rat and human [22] but also in other tissues such as brain cortex [23],

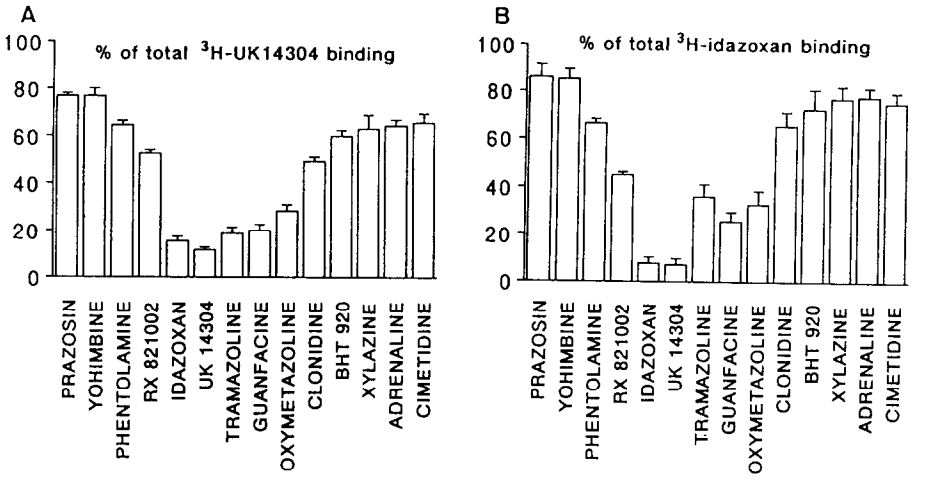

Fig. 3. Pharmacological profiles of [^{3}H]UK 14304 and [^{3}H]idazoxan binding on rat adipocyte membranes. (A) Total binding of 4–6 nM [^{3}H]UK 14304 and (B) 8–14 nM [^{3}H]idazoxan was competed by high concentrations (10^{-4} M) of various agents. Results are expressed in % of total binding. The residual binding on glass fiber filter alone represented in these conditions less than 2% of total binding for both radioligands. Each column represents means ± SE of 5–7 experiments carried out as described in Materials and Methods.

smooth muscle [11], platelets [22] and adipocytes [4, 14]. Thus we compared the [^{3}H]UK 14304 and [^{3}H]idazoxan binding characteristics in rat adipocytes in order to see if [^{3}H]UK 14304 binds to the same non-adrenergic sites as those labeled by [^{3}H]idazoxan. The rank order of the different competitors studied, based on their maximal displacing capacities, was for [^{3}H]idazoxan, comparable to that obtained with [^{3}H]UK 14304 (Fig. 3B) suggesting a similarity of the sites identified by both radioligands. Further support for the similarity between [^{3}H]idazoxan and [^{3}H]UK 14304 binding sites was the resemblance of the competition curves shown in Fig. 4. For both ligands, UK 14304 and idazoxan competition curves were characterized by IC_{50} values around 10 nM and by a complete inhibition of binding (90% inhibition achieved at 10^{-5} M) whereas IC_{50} values for adrenaline were in the micromolar range with maximal effects limited to at most 20% inhibition. RX 821002 only partially competed the non-adrenergic portion of [^{3}H]UK 14304 and [^{3}H]idazoxan binding since only 50% inhibition of total binding was reached at the highest concentration tested. Taken together, the data from Figs 3 and 4 support the view that [^{3}H]UK 14304, like [^{3}H]idazoxan, binds not only to α_2-adrenoceptors but also to non-adrenergic binding sites.

So, on fat cell membranes, the α_2-adrenergic sites were those displaced by adrenaline or BHT 920 whereas the non-adrenergic binding sites were not recognized by these drugs. The non-adrenergic sites appeared to be neither α_1-adrenergic (10^{-4} M prazosin displaced only about 20% of binding) nor histaminergic since they were not recognized by histamine (not shown) or the H2-antagonist cimetidine (Fig. 3). All the most potent competitors of the binding of both radioligands were imidazolidine derivatives: idazoxan, UK 14304, tramazoline and oxymetazoline. Thus, the non-adrenergic sites labeled by both radioligands appeared to exhibit, in rat adipocytes, a high affinity for imidazolinic compounds. However, some imidazolinic derivatives such as clonidine (α_2-agonist), phentolamine and RX 821002 (α_2-antagonists) shared only a poor affinity for these non-adrenergic sites. Moreover, other compounds containing an imidazole ring: cimetidine (H2-antagonist, Fig. 3), histamine and imidazole-4-acetic-acid (not shown) were totally inefficient in inhibiting [^{3}H]UK 14304 and [^{3}H]idazoxan binding. These data suggest that the presence of an imidazoline residue is necessary but not sufficient for the recognition abilities of these non-adrenergic sites. Moreover, the apparent high affinity of guanfacine (α_2-agonist containing a guanidine residue) for these sites allowed their comparison with the sites described on pig kidney membranes [12] which have a good affinity not only for imidazoline but also for guanidine derivatives. Still, they appeared different from the imidazole binding sites implicated in the hypotensive action of clonidine [24] since they were poorly affine for this drug. They also appeared to be different from the imidazoline binding sites described in the rabbit since, whatever the tissue considered [10, 11, 13, 14, 23], the imidazoline sites found in this species exhibited only low affinity for UK 14304. The non-adrenergic sites of the rat adipocyte thus shared the characteristics of the [^{3}H]idazoxan binding sites described in human, pig and rabbit kidneys and belongs to one of the three subtypes of imidazoline-preferring sites according to the recent classification of Michel and Insel [13].

Thus, it was of interest to know which kind of site is involved in the antilipolytic action of UK 14304: α_2-adrenoceptor or imidazoline-preferring site? It seems unlikely that non-adrenergic binding sites mediate the UK 14304 effect since (i) yohimbine, phentolamine and RX 821002, which did not interact with these sites (Fig. 3), blocked the 1 μM UK 14304-induced antilipolysis; (ii) imidazoline derivatives

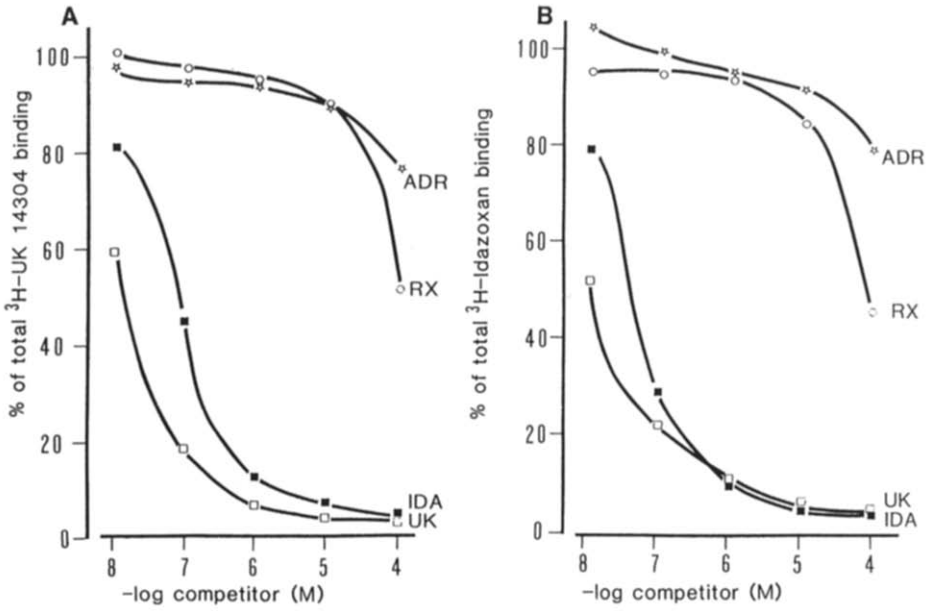

Fig. 4. Competition of [^{3}H]UK 14304 and [^{3}H]idazoxan binding. (A) Inhibition of the total binding of 10 nM [^{3}H]UK 14304, and (B) 14 nM [^{3}H]idazoxan, was studied in rat adipocyte membranes with the following competitors: IDA: idazoxan, UK: UK 14304, RX: RX 821002, ADR: adrenaline. Results are expressed as % of total radioligand bound. Each point represents the mean of three experiments. SE bars were deleted for clarity, they did not exceed 5%.

such as oxymetazoline, tramazoline (α_2-agonists) and idazoxan (α_2-antagonist), having a high affinity for the non-adrenergic sites (Fig. 3) did not induce an antilipolytic response similar to that of UK 14304 (Fig. 1); and (iii) BHT 920 did not bind to non-adrenergic sites but induced a significant antilipolytic effect. Moreover, in Zucker rat adipocytes, adrenaline itself (when its β-component is blocked by propranolol) induced an antilipolytic response [1]. Furthermore, to investigate the probable α_2-adrenergic nature of the receptor involved in the generation of UK 14304 effects it was necessary to quantify the rat adipocyte α_2-adrenoceptors correctly. This can be achieved by supressing the binding to these non-adrenergic sites (i) using a radioligand which has low affinity for these sites and/or (ii) by an accurate definition of the non-specific binding values, i.e. the binding non-displaceable by the physiological catecholamines.

α_2-Adrenoceptor identification by [^{3}H]*RX* 821002.

Among the α_2-adrenergic agents which exhibited weaker affinity for the non-adrenergic "imidazolinic" binding sites, it was of interest to test if those available under a tritiated form could provide suitable tools for the identification of rat fat cell α_2-adrenoceptors. For [^{3}H]yohimbine and [^{3}H]clonidine, the analysis of maximal displacing capacities of various competitors carried out as described above, showed that: (i) amounts of ligand bound were at the limits of detection and (ii) none of the agents tested displaced more than 40% of the total binding. Nevertheless, no striking differences in maximal inhibitions of binding were found (not shown) in contrast to the situation reported for [^{3}H]UK 14304 and [^{3}H]idazoxan. The poor characteristics of [^{3}H]clonidine and [^{3}H]yohimbine binding on rat adipocytes are in good agreement with previous studies describing lack of clonidine binding on rat white adipocytes [8] or weak affinity ($K_d \approx 20$ nM) of both radioligands in rat brown adipocytes [25, 26]. RX 821002, an idazoxan derivative has recently been described to be a potent α_2-antagonist [15]. Its use under a labeled form has only been reported, to our knowledge, on hamster [4] adipocytes and HT29 cells [27]. To examine whether [^{3}H]RX 821002 only binds to α_2-adrenergic sites, the same analysis as previously used for the other radioligands was undertaken: all adrenergic agents inhibited most of the [^{3}H]RX 821002 binding (Fig. 5). No difference between imidazolinic derivatives and non-imidazolinic drugs could be evidenced. All competitors—except cimetidine—achieved 60–80% inhibition of the total binding of relatively high concentrations of [^{3}H]RX 821002 (6 nM). Adrenaline itself (10^{-4} M) inhibited $68 \pm 3\%$ of total binding. [^{3}H]RX 821002 binding on histaminergic receptors was thus excluded and the non-adrenergic binding sites for imidazolinic derivatives were apparently not identified by this ligand. Furthermore, approximately 20% of total [^{3}H]RX 821002 binding in rat adipocytes was not competed for by any of the drugs tested and was, therefore, non-specific. In contrast to [^{3}H]UK 14304 and [^{3}H]idazoxan, the definition of specific [^{3}H]RX 821002 binding did not depend on the chemical nature of the adrenergic competitor. However, to avoid possible interaction with non-adrenergic sites, 200 μM-epinephrine (the physiological agonist) was used instead of 10^{-5} M phentolamine or idazoxan for the determination of non-specific binding in saturation experiments (Fig.

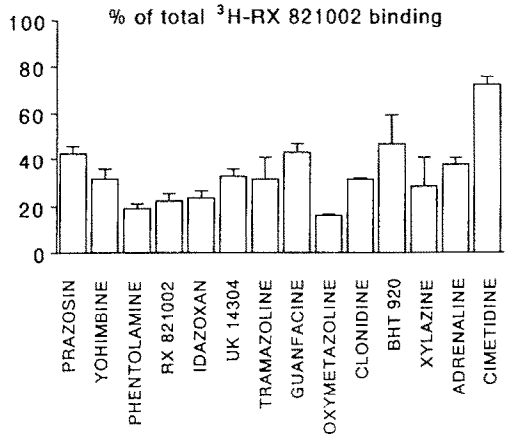

Fig. 5. Pharmacological profile of [³H]RX 821002 binding sites on rat adipocyte membranes. Total binding of [³H]RX 821002 (6–10 nM) was competed by 10^{-4} M of various agents. Results in % of total binding are means ± SE of 3–7 separate experiments. Residual binding on filters alone accounted for 9.9 ± 0.3% of total binding on rat adipocyte membranes which represented at 6 nM: 58 ± 8 fmol/mg protein.

6). In these conditions, the saturation curves showed that total binding of [³H]RX 821002 was four-fold less than that of [³H]UK 14304 (see Fig. 1). Non-specific binding was linear and represented about 30% of total binding at 2 nM. Scatchard analysis of six separate experiments gave B_{max} values of: 35 ± 2 fmol/mg protein with a K_d of 2.6 ± 0.6 nM. Scatchard analysis yielded a linear plot and Hill coefficients ranged from 0.949 to 1.024 (not different from unity), suggesting the presence of a single class of [³H]RX 821002 binding sites.

The competition curves of [³H]RX 821002 exhibited the classical pattern for the competition of the binding of an antagonist ligand: shallow curves for agonists (reflecting the presence of high and low affinity states of receptors, with pseudo Hill coefficients different from unity as reported for UK 14304 in Fig. 7A) and steeper curves for antagonists (illustrated by idazoxan and RX 821002 competition curves in Fig. 7B). The rank order of relative potencies, based on mean K_i values, was for agonists: UK 14304 > adrenaline > clonidine ⩾ oxymetazoline (mean K_i of three determinations: 11, 37, 41 and 54 nM, respectively). For antagonists, the rank order of relative potencies was: RX 821002 > idazoxan > phentolamine ⩾ prazosin (mean K_i of three determinations: 1, 19, 95 and 1850 nM, respectively. [³H]RX 821002 binding exhibited some stereoselectivity since competition studies with the (−) and the (+) isomers of adrenaline showed that (−)-adrenaline was more affine than the (+)-stereoisomer (respective apparent K_i values were: 43 ± 13 and 273 ± 156 nM, N = 3) whereas maximal competing capacity was identical for both isomers.

Since [³H]RX 821002 was the first radioligand found to exhibit high affinity and selectivity for the α_2-adrenoceptors of the rat adipocyte, an attempt to further characterize the α_2-adrenoceptors was undertaken.

Pharmacological subtype of rat adipocyte α_2-adrenergic receptors.

According to Bylund's classification [28] α_2-adrenoceptors can be divided into at least two subtypes according to their relative affinity for selective drugs such as oxymetazoline and prazosin. As oxymetazoline exhibited a higher affinity than prazosin (Fig. 7B) in inhibition of [³H]RX 821002 binding, the rat adipocyte α_2-adrenoceptor seems to belong to the α_2A subtype. However, this conclusion depends on the absence of subtype selectivity of [³H]RX 821002 which has only been shown to bind

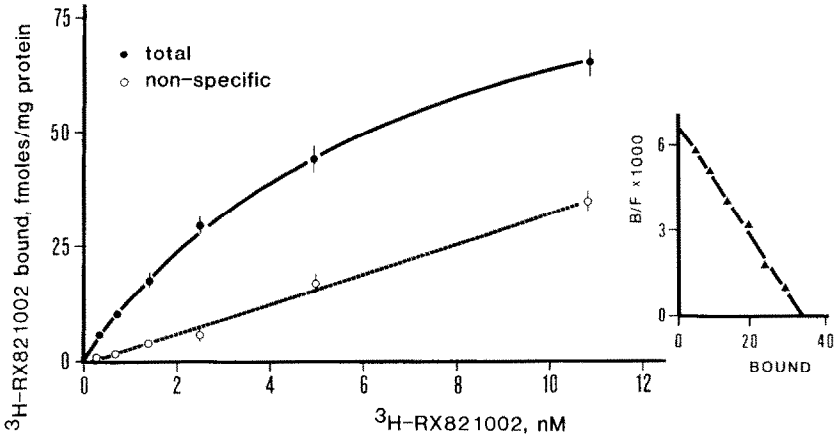

Fig. 6 [³H]RX 821002 binding on rat fat cell membranes. Crude membranes were incubated with increasing concentrations of [³H]RX 821002. Total (●) and non-specific (○) binding defined in the presence of 200 μM adrenaline, were determined after 30 min (incubation at 25°). Each point represents mean ± SE of six different batches of membranes. The binding parameters were obtained by Scatchard analysis. Inset: Scatchard plot of a representative experiment (B_{max} = 34 fmol/mg protein, K_d = 1.9 nM, n_H = 1.02). Mean values of six separate determinations were: B_{max} = 35 ± 2 fmol/mg protein, K_d = 2.6 ± 0.6 nM.

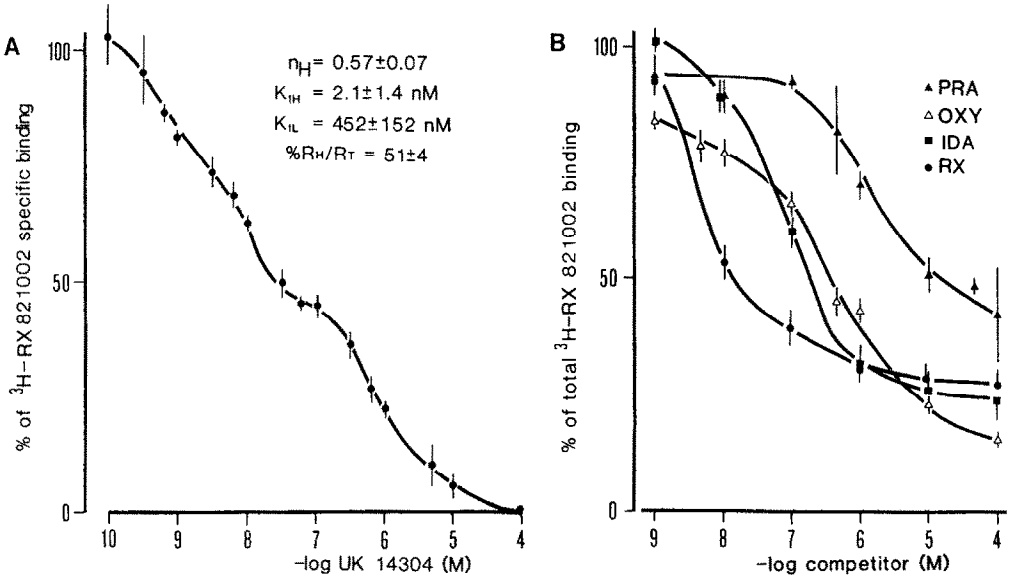

Fig. 7. Competition of [^{3}H]RX 821002 binding on rat adipocyte membranes. (A) Inhibition of [^{3}H]RX 821002 binding (5nM) was studied in the presence of UK 14304. Data are expressed in % of [^{3}H]RX 821002 specific binding using 10^{-4} M UK 14304 as reference. Residual binding in presence of 10^{-4} M UK 14304 represented 23 ± 5% of total binding. Means ± SE of four determinations. Individual competition curves were analysed using the EBDA-Ligand computer program; parameters for high- and low-affinity component are given in the inset. (B) Competition of total [^{3}H]RX 821002 binding by: PRA: prazosin, OXY: oxymetazoline, IDA: idazoxan, RX: RX 821002. Means ± SE of three determinations obtained in similar conditions to (A); mean K_i values were calculated according to the Cheng and Prusoff's equation, the values are given in the text.

correctly to the well documented α_2A receptor of HT29 cells [27] but not yet to α_2B or α_2C subtypes. Nevertheless, competition studies carried out on [^{3}H]yohimbine agree with the α_2-A nature of rat adipocyte receptors since oxymetazoline was shown to inhibit [^{3}H]yohimbine binding better then prazosin (30 vs 15% inhibition of total binding at 10^{-5} M competitor). However, no reliable K_i values could be calculated from these competition experiments (not shown) because of the reduced binding capacity, the poor affinity and the high level of non-specific binding of [^{3}H]yohimbine (not shown). These poor characteristics of [^{3}H]yohimbine binding on rat fat cells suggest that the adipocyte α_2-adrenoceptor is slightly different from the classical α_2A-adrenoceptors of the human platelet and HT29 cell since these receptor models do not have weak affinity for yohimbine. In fact, the ratios of prazosin IC_{50}/yohimbine IC_{50} in competition of UK 14304-induced antilipolysis (or [^{3}H]RX 821002 binding competition) which ranged around 20 in rat adipocytes, are strikingly lower than the 1000-fold difference found for the α_2A-receptor of the human fat cell (J. Galitzky, D. Larrouy, M. Lafontan and M. Berlan, unpublished results) or HT29 cell [27]. In addition, competition curve of prazosin (Fig. 7B) is rather shallow and contrasts with the steeper curve we obtained with the same drug on [^{3}H]RX 821002 binding on hamster adipocytes [4]. So further experiments are needed to completely characterize the subtype of the rat fat cell α_2-adrenoceptors. On the contrary, the non-adrenergic sites of rat adipocytes appeared to be similar to those reported in human kidney [22] or brain cortex [23]: in adipocytes, the K_i values of idazoxan (19 ± 3 nM) for inhibition of [^{3}H]RX 821002 binding were similar to those defined in [^{3}H]UK 14304 and [^{3}H]idazoxan competition studies (13 ± 4 and 12 ± 4 nM, respectively) indicating that idazoxan has similar affinities for the α_2-adrenoceptors and the non-adrenergic binding sites, a finding in good agreement with that described in the other models [13, 22, 23]. Finally, as shown in hamster adipocytes [4] and HT29 cells [27], [^{3}H]RX 821002 is a suitable tool for investigations on α_2-adrenoceptors even in tissues which possess imidazoline binding sites and α-receptors exhibiting low affinity for yohimbine.

In conclusion, the present findings show that: [^{3}H]UK 14304 not only binds to α_2-adrenoceptors but also to non-adrenergic binding sites located on rat adipocyte membranes which appear to be identical in nature to those identified by idazoxan on other cell models including hamster and rabbit adipocytes. However, these non-adrenergic sites are not directly involved in the antilipolytic action of UK 14304 since (i) α_2-antagonists blocked UK 14304-induced antilipolysis even if they poorly interacted with such sites (yohimbine, RX 821002, phentolamine); (ii) idazoxan, which recognized these non-adrenergic sites, shared the same blocking properties as the other α_2-antagonists: and (iii) other imidazoline derivatives, which are α_2-agonists like UK 14304 but with lower α_2-adrenergic intrinsic activity, were weak antilipolytic agents. In addition, the α_2-adrenergic nature of the UK 14304 antilipolytic effect is supported by the findings of Garcia-Sainz and Martinez

[29] who demonstrated that the UK 14304-induced inhibition of cyclic AMP accumulation is in rat adipocytes totally reversed by yohimbine, a non-imidazoline α_2-blocking agent.

The α_2-antagonist [^{3}H]RX 821002 provides a suitable tool for the identification of α_2-adrenoceptors on rat adipocytes since it did not interact with non-adrenergic binding sites for imidazolinic compounds and exhibited higher affinity and saturable binding. Moreover, RX 821002 possessed the best α_2-blocking properties among the α_2-antagonists tested. The present results and those of other studies from the laboratory allow a comparison to be made between the α_2-adrenoceptor equipment of the rat adipocyte and that of other species like human [3], (J. Galitzky, D. Larrouy, M. Lafontan and M. Berlan, unpublished results) and hamster [4]. The α_2-adrenoceptors of the rat adipocyte appear to be less numerous than those described in other species when quantified with [^{3}H]RX 821002 (B_{max} = 810 ± 78, 776 ± 60 and 35 ± 2 fmol/mg protein in human, hamster and rat) or with [^{3}H]UK 14304 when binding at the non-adrenergic component is precluded (B_{max} = 865 ± 62, 438 ± 40 and 16 ± 5 fmol/mg protein, respectively). They are also slightly different in their pharmacological properties (unresponsiveness to clonidine, low affinity for yohimbine). It should be noted that the reduced α_2-adrenergic receptivity of rat adipocyte is linked to a reduced antilipolytic efficiency of α_2-agonists. It can be concluded that rat adipocytes possess functional α_2-adrenoceptors able, when stimulated by agonists, to induce an antilipolysis. However, they are less numerous and slightly different from the α_2-adrenoceptors of the adipocytes of other species.

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