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Binding of [^{3}H]Idazoxan and of Its Methoxy Derivative [^{3}H] RX821002 in Human Fat Cells: [^{3}H]Idazoxan but Not [^{3}H] RX821002 Labels Additional Non-α_2-Adrenergic Binding Sites

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SUMMARY

Binding studies were carried out in human fat cell membranes with two α_2-adrenergic antagonists, [^{3}H]idazoxan and its methoxy derivative [^{3}H]RX821002. Inhibition studies with epinephrine enantiomers indicate that [^{3}H]RX821002 only binds to α_2-adrenoceptors, whereas [^{3}H]idazoxan labels α_2-adrenoceptors and additional nonadrenergic sites (NAIBS). NAIBS and α_2-adrenoceptors display different affinities towards drugs from various chemical families. Imidazoline and some guanidine derivatives exhibit a high affinity for NAIBS. Pharmacological studies of human NAIBS indicate that they are slightly different from those previously reported in the rabbit, suggesting the existence of several subtypes of NAIBS. Furthermore, NAIBS are different from the previously described "imidazoline-preferring sites." [^{3}H] idazoxan and [^{3}H]RX821002 saturation analyses were performed in human adipocytes from different anatomical locations, in order to compare the number of NAIBS and α_2-adrenoceptors. Although there was an important variation in NAIBS and α_2-adrenoceptor numbers in the studied samples, a very poor correlation was obtained between the B_{max} values of the two sites. Moreover, alkylation of α_2-adrenoceptors by phenoxybenzamine produces a 90% reduction in accessible [^{3}H]RX821002 binding sites, without modification of [^{3}H]idazoxan binding. These data show that NAIBS are not closely related to the α_2-adrenergic molecule. In addition, benextramine appears to be a reversible competitor at NAIBS. [^{3}H]Idazoxan binding, but not [^{3}H]RX821002 binding, is sensitive to K^+, suggesting that the domains involved in the ligand-NAIBS interaction are different from those involved in the ligand-α_2-adrenoceptor interaction.

Human fat cells are known to present a dual adrenergic receptivity, which mediates the antagonistic effects of catecholamines. Whereas β-adrenoceptors mediate the lipolytic action of epinephrine via an increase of intracellular cyclic AMP, α_2-adrenoceptors are responsible for its antilipolytic action by counteracting the accumulation of this second messenger (1, 2). The functional response of human fat deposits to epinephrine differs according to their location (3). As previously shown, these differences may be correlated with variations in the α_2-/β-adrenoceptor number ratio, due to changes in α_2-adrenoceptor density, which suggests that this inhibitory receptor plays a local role in human adipose tissue function (4).

Clearly, such studies of the α_2-/β-adrenoceptor balance necessitate an accurate determination of the α_2-adrenoceptor number. Among the different α_2-antagonist radioligands that can be used for such a purpose, [^{3}H]idazoxan ([^{3}H]RX781094) was considered to be one of the most α_2-selective tools available (5). Very recent reports indicate, however, that this probe must be used cautiously, because [^{3}H]idazoxan, in addition to binding to α_2-adrenoceptors, also binds with high affinity at NAIBS in several tissues (6–10). To date, the role of these NAIBS is totally unknown. It must be noted, however, that a purified brain extract has been shown to compete for NAIBS in rabbit kidney. This extract, termed "clonidine-displacing substance," may represent a possible endogenous ligand for these sites (11).

Because binding data have suggested that NAIBS and α_2-adrenoceptors might be closely related in rat and human tissues (10) and because the presence of NAIBS was also reported in rabbit adipose tissue (9), we decided to look at the possible existence of NAIBS in human adipocytes. Compared with previously used tissue homogenates, isolated fat cells offer at least two advantages, (i) they constitute a homogeneous preparation where the properties of both NAIBS and α_2-adrenoceptors can be compared and (ii) as mentioned above they possess variable amounts of α_2-adrenoceptors, making a parallel study of α_2-adrenoceptor and NAIBS density possible. For these reasons, an extensive study of NAIBS was attempted in this model.

In the present work, we report on the binding of [^{3}H]idazoxan and of its methoxy derivative, [^{3}H]RX821002. The structures

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ABBREVIATIONS: NAIBS, nonadrenergic idazoxan binding sites; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

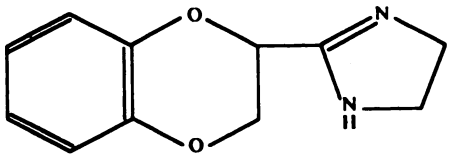
of the radioligands are presented in Fig. 1. The data clearly demonstrate that [³H]RX821002 binds only to α_2-adrenoceptors, whereas [³H]idazoxan labels α_2-adrenoceptors and NAIBS. The two binding entities display different affinities towards drugs from various chemical families and different sensitivities to cations. Utilization of irreversible adrenergic blockers suggests that the binding moieties of the two sites are different. Moreover, NAIBS number and α_2-adrenoceptor number appear to be unrelated. These results support the idea that α_2-adrenoceptors and NAIBS are independent entities.

Materials and Methods

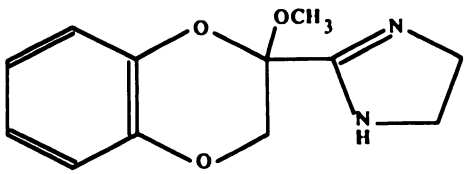
Isolation of human adipocytes and preparation of fat cell ghosts. Adipose tissue samples were obtained from premenauposal women undergoing surgical treatment. The patients were healthy and none had any identified metabolic or endocrinological disorders. After surgical excision, the adipose tissue was quickly transported to the laboratory in sterile physiological saline (0.9% NaCl, 5 mM HEPES, 6 mM glucose, pH 7.5). Adipocytes were isolated according to the method of Rodbell (12), with minor modifications. The adipose tissue was cut into small pieces and incubated for 30–40 min at 37°, with vigorous shaking, in Krebs Ringer bicarbonate buffer (pH 7.5) containing 35 mg/ml bovine serum albumin, 6 mM glucose (KRBA buffer), and 1 mg/ml collagenase. After collagenase digestion, the adipocytes were separated from the stromavascular fraction by floatation and washed three times in KRBA buffer.

Crude membrane preparation. Crude membranes were prepared by lysis of the adipocytes in a hypotonic medium containing 2 mM Tris·HCl, 2.5 mM $MgCl_2$, 1 mM $KHCO_3$, 100 μM EGTA (35 mOsM, pH 7.5), and the following protease inhibitors: leupeptin (1 μg/ml), benzamidine (0.1 mM), and phenylmethylsulfonyl fluoride (100 μM). Lysis of adipocytes was performed at 20–22° in order to minimize trapping of membranes in the coalescing fat cake. Cell ghosts were separated from the fat cake by centrifugation (40,000 × g, 15 min at 20°). The pellet was resuspended in 1 ml of lysing medium and immediately frozen at −80° until use.

Thawed crude membranes were diluted in a large volume (30 ml) of 50 mM Tris·HCl, 5 mM EDTA, pH 7.4, and centrifuged (40,000 × g, 10 min at 4°). The pellet was washed once in Tris-Mg^{2+} buffer (50 mM Tris·HCl, 0.5 mM $MgCl_2$, pH 7.5), followed by a second centrifugation. The resulting pellet was finally resuspended in the required volume of Tris-Mg^{2+} buffer (protein concentration ranging from 1 to 1.5 mg/ml) and immediately used for measurements of binding capacity. In studies dealing with the effect of ions, Mg^{2+} was omitted from the washing buffer and the membrane incubation medium.



IDAZOXAN



RX821002

Fig. 1. Chemical structures of idazoxan (RX781094) and RX821002.

Binding studies. Binding studies were performed at 25° for 45 min; steady states were achieved for the three radioligands by this time. The incubation medium consisted of 100 μl of radioligand and 100 μl of membrane suspension, made up to a final volume of 400 μl with Tris-Mg^{2+} buffer. At the end of the incubation period, the suspensions were filtered through GF/C Whatman filters, using a 12-sample Skatron cell harvester. The tubes and filters were washed with 20 ml of washing buffer (0.5 mM $MgCl_2$ or 10 mM Tris·HCl). The radioactivity retained on the filters was determined in presence of 2 ml of scintillation mixture (Emulsifier Safe Packard), using a scintillation counter (Packard) with an efficiency of 45–50%.

The specific binding was calculated as the difference between total and nonspecific binding, which was defined in the presence of 100 μM naphazoline for [³H]idazoxan and 100 μM epinephrine for [³H]RX821002.

For saturation studies, radioligand concentrations ranged from 0.2 to 10 nM for [³H]RX821002 and from 0.5 to 30 nM for [³H]idazoxan. The dissociation constant (K_D) and the maximum number of binding sites (B_{max}) were determined from equilibrium data, using a computer-assisted linear transformation of the saturation isotherm (13). For competition studies, various concentrations of competing drug were added to the incubation mixture before addition of the membrane preparation. The data were analyzed using INHIBITION, a computer program allowing curve-fitting to a one-site inhibition model (13). Apparent inhibition constants (K_i) were calculated according to the Cheng and Prusoff equation (14). Protein determination was measured according to the method of Lowry *et al.* (15), using bovine serum albumin as standard. A paired t test was used to compare the binding parameters in parallel studies. The values are means ± standard errors.

Receptor blockade with benextramine and phenoxybenzamine. Human fat cell membranes (5 mg of protein) were incubated for 15 min in 5 ml of Tris-Mg^{2+} buffer alone or containing 5×10^{-5} M benextramine or 10^{-5} M phenoxybenzamine. Membranes were then washed three times with 30 ml of Tris-Mg^{2+} buffer, at intervals of 15 min between washings, in order to remove surplus drugs. At the end of the washing procedure, the membranes were resuspended in Tris-Mg^{2+} buffer for saturation analysis with [³H]idazoxan and [³H]RX821002.

Drugs and chemicals. [³H]RX821002 (43.8 Ci/mmol) was a generous gift from Reckitt and Colman (Kingston-upon-Hull, UK). [³H]UK14304 (82.7 Ci/mmol) and [³H]idazoxan (41 Ci/mmol) were obtained from New England Nuclear (Boston, MA) and from Amersham (Les Ulis, France), respectively.

RX821002, efaroxan, RX791050, RX801080, and idazoxan (16–18) were provided by Reckitt and Colman. Yohimbine, (−)-epinephrine, imidazole-4-acetic acid, histamine, oxymetazoline, naphazoline, and amiloride were purchased from Sigma Chemical Co. (St. Louis, MO). (+)-Epinephrine and Winthrop derivatives (19) were from Sterling-Winthrop (Rensselaer, NY). Prazosin, UK14304, guanoxan, and guanethidine were from Pfizer (Sandwich, UK). Guanabenz was from Sandoz (Basel, Switzerland), clonidine from Boehringer (Ingelheim, FRG), phentolamine and CGP12177 from Ciba-Geigy (Basel, Switzerland), and rauwolscine from Extrasynthèse (Genay, France). Rilmenidine was a generous gift from Servier Laboratories (Paris, France). Phenoxybenzamine, SKF104078, and SKF86466 were obtained from Smith Kline and French (Philadelphia, PA) and benextramine from Aldrich Chemical Co. (Strasbourg, France). Collagenase came from Boehringer. All other chemicals were reagent grade of the best quality available.

Results

Inhibition of [³H]RX821002, [³H]UK14304, and [³H]idazoxan binding by idazoxan, yohimbine, and (−)- and (+)-epinephrine. One of the criteria for the reliability of adrenoceptor radioligand binding data is stereoselectivity towards catecholamine isomers, i.e., (−)-epinephrine is more potent than its enantiomer (+)-epinephrine in competition

studies. We, therefore, studied the inhibition of [^{3}H]RX821002, [^{3}H]idazoxan, and [^{3}H]UK14304 binding by yohimbine, idazoxan, and (−)- and (+)-epinephrine (Fig. 2). From 10^{-11} to 10^{-3} M, (−)-epinephrine was always more potent than (+)-epinephrine in inhibiting [^{3}H]RX821002 binding. Moreover, both idazoxan and yohimbine competed with high affinity for [^{3}H]RX821002 binding sites. At the higher concentrations of both antagonists, residual binding of [^{3}H]RX821002 was identical and very low (3–5% of total), demonstrating that it only labeled the α_2-adrenoceptor (Fig. 2A). In contrast, epinephrine enantiomers displayed stereoselectivity towards [^{3}H]idazoxan and [^{3}H]UK14304 binding sites at concentrations below 10^{-5} M but not at higher concentrations (Fig. 2, B and C). In addition, for both imidazoline radioligands, inhibition curves of yohimbine were biphasic, making it clear that [^{3}H]idazoxan and [^{3}H]UK14304 labeled both α_2-adrenoceptors and additional nonstereoselective binding sites. As shown by its monophasic competition curves, idazoxan was able to totally inhibit the binding of both radioligands, suggesting that it is not discriminative towards the two sites and that the additional nonadrenergic sites display a high affinity for idazoxan (NAIBS).

Comparative study of [^{3}H]RX821002 and [^{3}H]idazoxan saturation binding. [^{3}H]Idazoxan and [^{3}H]RX821002 binding curves are depicted in Fig. 3. Saturation curves of [^{3}H]idazoxan binding in human fat cells were studied in order to discriminate between the binding of this radioligand to NAIBS and to α_2-adrenoceptors (Fig. 3A). In the presence of 10^{-6} M yohimbine, an α_2-adrenergic (but not imidazoline) compound, the [^{3}H]idazoxan binding did not increase linearly. This curve is, in fact, the nonspecific binding curve for α_2-site delineation or the total binding curve for NAIBS definition. When 10^{-4} M naphazoline, a combined α_2-adrenergic and imidazoline drug,

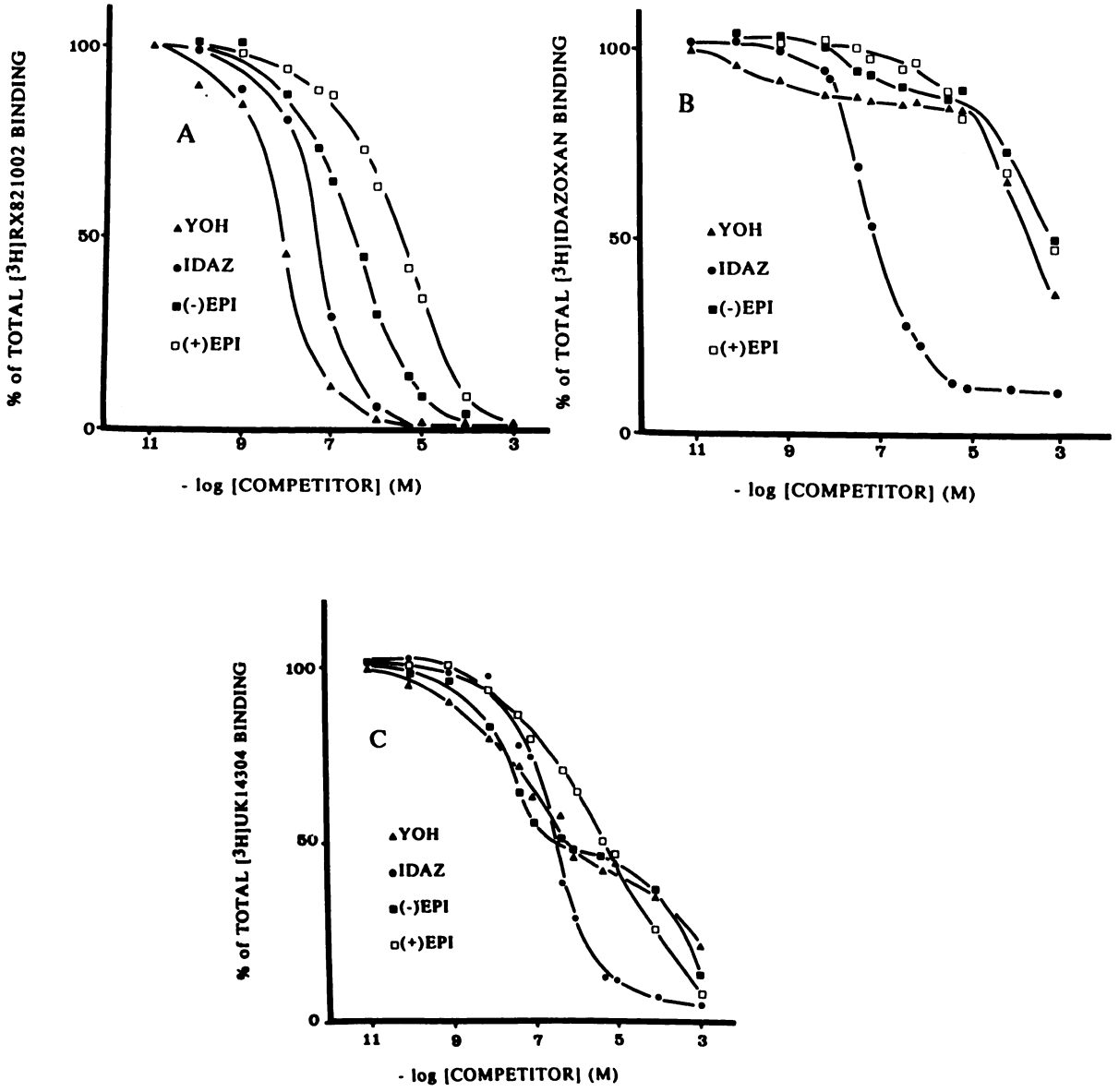


Fig. 2. Inhibition of [^{3}H]RX821002 (A), [^{3}H]idazoxan (B), and [^{3}H]UK14304 (C) binding by idazoxan (●), yohimbine (▲), (+)-epinephrine (□), and (−)-epinephrine (■). Inhibition studies were performed in the presence of 18–20 nM [^{3}H]idazoxan, 1–1.5 nM [^{3}H]RX821002, or 4–5 nM [^{3}H]UK14304. The data are expressed as the percentage of total ^{3}Hradioligand bound observed in the absence of competitor. The data from this typical experiment are representative of those obtained in two others.

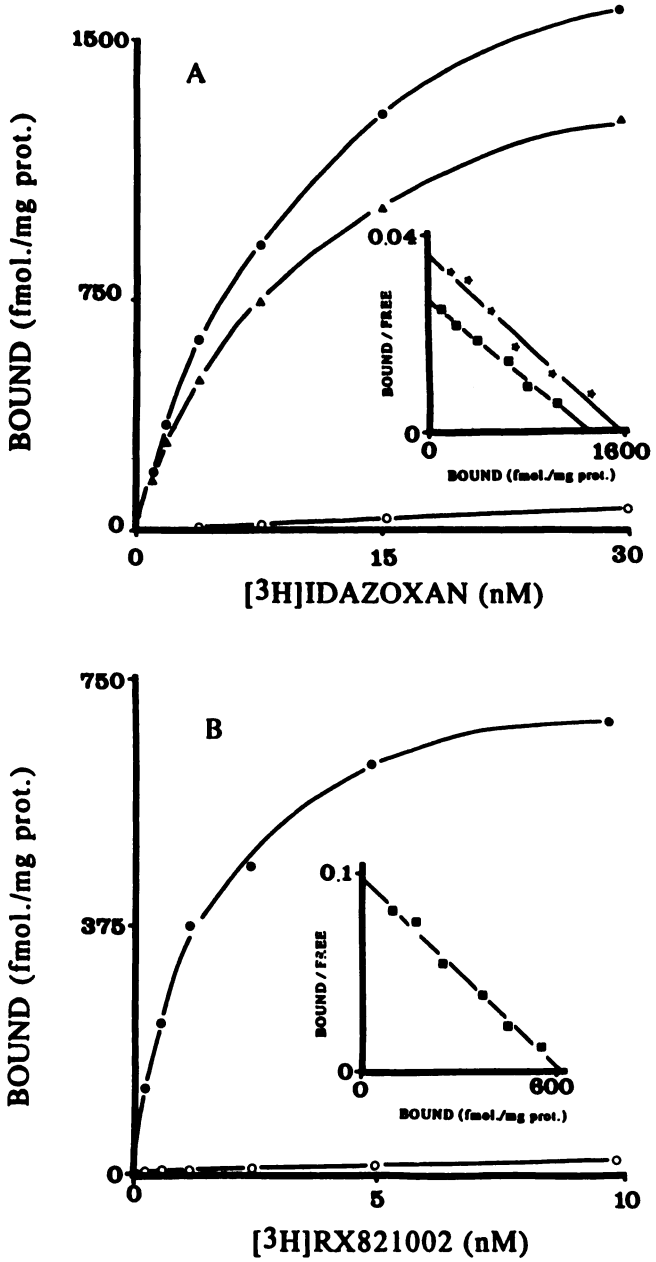


Fig. 3. Evolution of [^{3}H]idazoxan (A) and [^{3}H]RX821002 (B) binding. Human fat cell membranes were incubated for 45 min at 25° with different concentrations of [^{3}H]idazoxan (0.5 to 30 nM) and [^{3}H]RX821002 (0.2 to 10 nM). This experiment is representative of three others. A, Binding in the absence of competitor (●), in the presence of 10^{-6} M yohimbine (▲), and in the presence of 10^{-4} M naphazoline (○) are plotted as a function of free [^{3}H]idazoxan concentration. *Inset*, Scatchard plots from this specific experiment. Specific binding was calculated either as the data in the absence of competitor minus the data in the presence of 10^{-4} M naphazoline (α_2-sites plus NAIBS) or as the data in the presence of 10^{-6} yohimbine minus the data in the presence of 10^{-4} M naphazoline (NAIBS). Linear regression analysis of the first calculation (*) gave the following parameters for the binding of [^{3}H]idazoxan to α_2-adrenoceptors and NAIBS: K_D = 7.66 nM, B_{max} = 1574 fmol/mg of protein, and n_H = 0.95. Linear regression analysis of the second calculation (■) gave the following parameters for the binding of [^{3}H]idazoxan to NAIBS alone: K_D = 8.70 nM, B_{max} = 1323 fmol/mg of protein, and n_H = 1.02. B, Binding in the absence of competitor (●) and in presence of 10^{-4} M (−)-epinephrine (○) is plotted as a function of free [^{3}H]RX821002 concentration. Linear regression analysis of the Scatchard plot (■) from this specific experiment gave the following binding parameters: K_D = 1.59 nM, B_{max} = 616.2 fmol/mg of protein, and n_H = 1.00.

was added, [^{3}H]idazoxan binding increased linearly with radioligand concentration, because naphazoline competed with both kinds of sites (α_2-sites and NAIBS). Hence, Scatchard transformations of the saturation isotherms can be obtained (i) for the quantification of both NAIBS and α_2-adrenoceptors, by measurement of the total [^{3}H]idazoxan binding minus the binding obtained in the presence of 10^{-4} M naphazoline or (ii) for the quantification of NAIBS alone, by measurement of [^{3}H]idazoxan binding in the presence of 10^{-6} M yohimbine (which masked the α_2-adrenoceptors) minus its binding in the presence of 10^{-4} M naphazoline. In both cases, Scatchard plots were linear and calculated Hill coefficients were close to unity, suggesting that the dissociation constants of [^{3}H]idazoxan for NAIBS and α_2-adrenoceptors were not very different (Fig. 3A).

Data obtained with [^{3}H]RX821002 are presented in Fig. 3B. Nonspecific binding, defined in the presence of 10^{-4} M epinephrine, increased linearly with radioligand concentration. Whichever competitor was used, the percentage of nonspecific binding was similarly low (1–2% at 1 nM [^{3}H]RX821002).

The results from the analyses of the saturation isotherms of both radioligands are summarized in Table 1. [^{3}H]RX821002 and [^{3}H]idazoxan (in the presence of 10^{-6} M yohimbine) labeled a single class of binding sites and the Hill coefficients calculated were not significantly different from unity. K_D values for [^{3}H]RX821002 at α_2-adrenoceptors and [^{3}H]idazoxan at NAIBS were 1.34 ± 0.11 and 11.34 ± 1.18 nM, respectively.

It was recently demonstrated that the density of α_2-adrenoceptors varies according to the location of the fat deposits in humans (4). So, we wanted to see whether a correlation existed between the number of α_2-adrenoceptors and the number of NAIBS in a situation where the number of α_2-adrenoceptors varied to a large extent. Nineteen different samples from omental, femoral, and abdominal fat deposits were randomly chosen and comparative saturation analyses were performed. B_{max} values for α_2-adrenoceptors and NAIBS varied from 167 to 780 fmol/mg of protein and from 488 to 1955 fmol/mg of protein, respectively. K_D values for both sites were in the same range as those calculated for abdominal fat cells (Table 1). The value of the NAIBS/α_2-adrenoceptor number ratio varied from 1.2 to 6.0, with a mean value of 2.7 ± 0.3. There was no significant correlation (r = +0.23, p = 0.40) between the two B_{max} values,

TABLE 1

Comparison of [^{3}H]idazoxan and [^{3}H]RX821002 saturation binding parameters in human fat cell membranes

Human fat cell membranes were incubated for 45 min at 25° with different concentrations of [^{3}H]idazoxan (0.5 to 30 nM) and [^{3}H]RX821002 (0.2 to 10 nM). For [^{3}H]idazoxan, specific binding was defined as the difference between binding in the presence of 10^{-6} M yohimbine (total nonadrenergic binding) and 10^{-4} M naphazoline (nonspecific binding). For [^{3}H]RX821002, nonspecific binding was determined using 10^{-4} M (−)-epinephrine. The dissociation constant (K_D) and the maximum number of sites (B_{max}) were calculated from equilibrium data using a computer-assisted linear transformation of the saturation isotherm (13). Results are means ± standard errors.

Radioligand	K_D	B_{max}	n_H
	nM	fmol/mg of protein	
Abdominal fat deposit[a]			
[^{3}H]Idazoxan	11.34 ± 1.18	1599 ± 132	0.99 ± 0.01
[^{3}H]RX821002	1.34 ± 0.11	566 ± 63	1.01 ± 0.01
Pooled fat deposits[b]			
[^{3}H]Idazoxan	11.02 ± 0.59	1054 ± 101	0.99 ± 0.01
[^{3}H]RX821002	2.87 ± 0.11	440 ± 42	1.01 ± 0.01

[a] Six experiments.
[b] Nineteen experiments.

suggesting that α_2-adrenoceptors and NAIBS are independent variables.

Specificity of [^{3}H]idazoxan and [^{3}H]RX821002 binding. [^{3}H]RX821002 specificity was compared with that of [^{3}H]idazoxan by testing the ability of various compounds from different chemical families to impair the binding of the two radioligands. In order to clearly compare NAIBS and α_2-adrenoceptors, all further experiments dealing with [^{3}H]idazoxan binding were performed in the presence of 10^{-6} M yohimbine. At this yohimbine concentration, [^{3}H]idazoxan will not interfere with the α_2-adrenoceptor, allowing a more accurate study of the characteristics of [^{3}H]idazoxan-NAIBS interactions (Fig. 2B). Apparent K_i values were determined, in order to calculate the affinity ratio of the drugs for NAIBS and α_2-adrenoceptors (Tables 2 and 3).

Imidazoline compounds did not exhibit the same rank order of potency towards the two binding sites (Table 2). Cirazoline, RX791050, and Winthrop derivatives displayed a higher affinity for NAIBS than for α_2-adrenoceptors. RX821002, phentolamine, clonidine, and efaroxan competed preferentially with α_2-adrenoceptors, whereas idazoxan was approximately equipotent at the two sites. Three derivatives of idazoxan and two related compounds were also tested. RX821002 is the 2-methoxy derivative of idazoxan (Fig. 1); efaroxan is the 2-ethyl derivative of RX801080. Substitution in position 2 enhanced (RX821002 versus idazoxan) or did not change (efaroxan versus RX801080) the α_2 affinity but dramatically decreased the affinity for NAIBS. Substitution on the imidazoline ring (RX791050) resulted in a decreased affinity for α_2-adrenoceptors.

The α_2-adrenergic full agonist of the imidazoline family, UK14304, exhibited a high affinity for α_2-adrenoceptors (K_i = 17.2 ± 3.5 nM, five experiments), with a pseudo-Hill coefficient different from unity (n_H = 0.61, five experiments). As expected from the [^{3}H]UK14304 inhibition data (Fig. 2C) reported above, this compound also exhibited a rather high affinity for NAIBS (K_i = 53.4 ± 6.8 nM, five experiments). Suprisingly, the pseudo-Hill coefficient for this site was also significantly different from unity (n_H = 0.57, five experiments). UK14304 competition studies on [^{3}H]RX821002 and [^{3}H]idazoxan binding with and without 10^{-3} M GTP indicated that, as expected for the α_2-adrenoceptors, addition of GTP modified the shape of the UK14304 competition curve (not shown) and increased pseudo-Hill coefficients (from n_H = 0.66 ± 0.02 to n_H = 0.80 ± 0.01, three experiments); by contrast, pseudo-Hill coefficients were unchanged for [^{3}H]idazoxan binding in the presence of 10^{-3} M GTP (n_H = 0.68 ± 0.04 n_H = 0.67 ± 0.01, three experiments). The lack of influence of GTP on the binding of UK14304 at NAIBS was confirmed, as shown in Fig. 4. [^{3}H]UK14304 binding at high affinity state α_2-adrenoceptors but not at NAIBS was sensitive to increasing concentrations of GTP. ATP had no effect on either site (not shown).

Other chemical families were tested in competition studies (Table 3). Rauwolscine and yohimbine, which did not inhibit [^{3}H]idazoxan binding, exhibited a similar high affinity at [^{3}H]RX821002 binding sites. Moreover, oxymetazoline was much more potent than prazosin. Taken together, these data confirm the α_{2A}-subtype of the human fat cell α_2-adrenoceptor, as defined by Bylund (20).

Other α_2-adrenergic compounds, such as guanidine derivatives (guanoxan and guanabenz), an oxazoline derivative (rilmenidine), and benzazepine derivatives (SKF86466 and SKF104078), competed with [^{3}H]idazoxan binding. Amiloride exhibited a higher affinity for NAIBS than for α_2-adrenoceptors. This demonstrated that nonimidazoline compounds were also able to compete with NAIBS.

Effect of metal ions on the binding of [^{3}H]idazoxan and [^{3}H]RX821002. Metal ions are known to modify the binding characteristics of radioligands, especially [^{3}H]idazoxan binding (21). This part of the study was designed to investigate the effect of changing the ion concentration in the incubation medium on [^{3}H]idazoxan binding.

Addition of $CaCl_2$ or $MgCl_2$ (0.01–10 mM) did not modify [^{3}H]idazoxan or [^{3}H]RX821002 binding. Monovalent cations (Na^+, Li^+, or K^+) were also ineffective in modifying [^{3}H]RX821002 binding but provoked a decrease in that of [^{3}H]idazoxan, with the following rank order of potency: $K^+ > Li^+ > Na^+$ (Fig. 5). At 100 mM, the percentage of inhibition reached

TABLE 2

Inhibition constants (K_i) of imidazoline compounds at [^{3}H]RX821002 (K_{i_1}) and [^{3}H]idazoxan (K_{i_2}) binding sites

Inhibition studies were performed in the presence of 11–12 nM [^{3}H]idazoxan or 1–1.5 nM [^{3}H]RX821002 and increasing concentrations of different competitors (10^{-11} to 10^{-3} M). In order to prevent binding of [^{3}H]idazoxan to α_2-adrenoceptors, [^{3}H]idazoxan binding studies were performed in the presence of 10^{-6} M yohimbine. The inhibition data were analyzed using INHIBITION, a computerized program for curve fitting to a one-site inhibition model (13). Inhibition constants (K_i) were calculated according to the Cheng and Prusoff equation (14). Results are means ± standard errors from more than three separate experiments.

Competitor	[^{3}H]RX821002		[^{3}H]idazoxan		K_{i_1}/K_{i_2}
	K_{i_1}	n_{H_1}	K_{i_2}	n_{H_2}	
	nM		nM		
Win52401	1,303 ± 25	1.08	20.8 ± 2.5	0.67	62.6
Win50478	5,697 ± 306	0.87	.248 ± 23	1.04	23.0
Cirazoline	193 ± 7	1.06	8.5 ± 2.7	0.88	22.8
RX791050	6,261 ± 1,129	0.95	335 ± 44	0.65	18.7
Idazoxan	19.0 ± 1.7	0.99	12.6 ± 1.6	0.93	1.5
Naphazoline	32.0 ± 11.8	0.82	68.2 ± 20.4	0.81	0.47
UK14304	17.2 ± 3.5	0.61	53.4 ± 6.8	0.57	0.32
RX801080	8.8 ± 3.0	0.99	110 ± 24	0.78	0.08
Oxymetazoline	16.3 ± 0.6	0.86	1,115 ± 7	0.85	0.015
Clonidine	32.2 ± 3.8	0.71	19,054 ± 3,796	ND[a]	0.002
Phentolamine	11.5 ± 2.7	0.82	34,728 ± 10,711	ND	<0.001
Efaroxan	6.1 ± 2.1	0.93	32,866 ± 2,679	ND	<0.001
RX821002	1.4 ± 0.5	1.00	10,681 ± 4,215	ND	<0.001

[a] ND, not determined.

TABLE 3

Inhibition constants (K_i) of nonimidazoline compounds at [³H]RX821002 (K_{i_1}) and [³H]idazoxan (K_{i_2}) binding sites

Inhibition studies were performed in the presence of 11–12 nM [³H]idazoxan or 1–1.5 nM [³H]RX821002 and increasing concentrations of different competitors (10^{-11} to 10^{-3} M). In order to prevent binding of [³H]idazoxan to α_2-adrenoceptors, [³H]idazoxan binding studies were performed in the presence of 10^{-6} M yohimbine. The inhibition data were analyzed using INHIBITION, a computerized program for curve fitting to a one-site inhibition model (13). Inhibition constants (K_i) were calculated according to the Cheng and Prusoff equation (14). Results are means ± standard errors from more than three separate experiments.

Competitor	[³H]RX821002		[³H]idazoxan		K_{i_1}/K_{i_2}
	K_{i_1}	n_{H_1}	K_{i_2}	n_{H_2}	
	nM		nM		
Amiloride	32,136 ± 6,779	ND[a]	3,873 ± 962	0.87	8.30
Histamine	34,161 ± 1,702	ND	7,442 ± 642	ND	4.59
Guanethidine	47,856 ± 5,633	ND	21,536 ± 1,395	ND	2.22
Guanoxan	94.0 ± 8.4	0.63	45.9 ± 17.1	0.51	2.05
Guanabenz	38.9 ± 4.5	0.64	29.9 ± 3.7	0.59	1.30
Rilmenidine	200 ± 32	0.67	1,931 ± 141	0.60	0.10
Prazosin	2,237 ± 409	1.06	61,244 ± 2,341	ND	0.037
SKF104078	366 ± 86	1.01	11,973 ± 858	ND	0.031
SKF86466	26.3 ± 0.2	0.97	7,659 ± 2,328	1.11	0.003
Rauwolscine	3.8 ± 0.3	0.90	>100,000	ND	<0.001
Yohimbine	3.0 ± 0.2	0.90	>100,000	ND	<0.001
CGP12177	64,360 ± 3,269	ND	>100,000	ND	ND
Imidazole-4-acetic acid	>100,000	ND	>100,000	ND	ND

[a] ND, not determined.

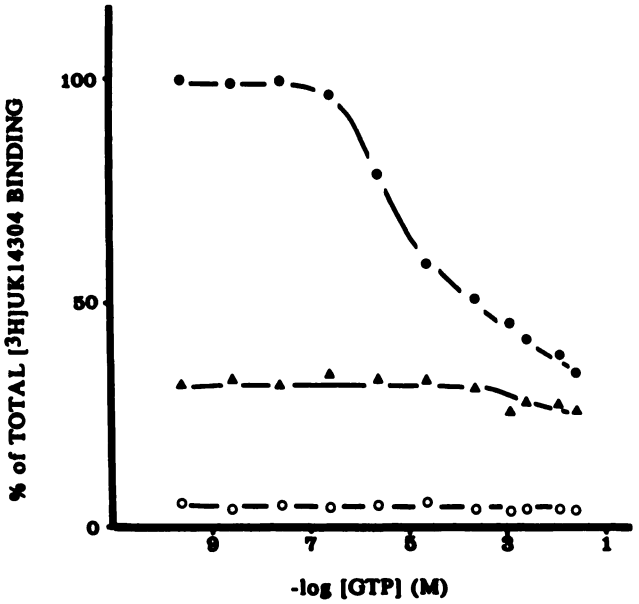


Fig. 4. Inhibition of [³H]UK14304 binding by GTP in the presence of buffer alone (●), 10^{-6} M yohimbine (▲), or 10^{-4} M naphazoline (○). Inhibition studies were performed in the presence of 4–5 nM [³H]UK14304. The data are expressed as a percentage of total [³H]UK14304 bound in the absence of competitor. The data from this typical experiment are representative of those obtained in three others.

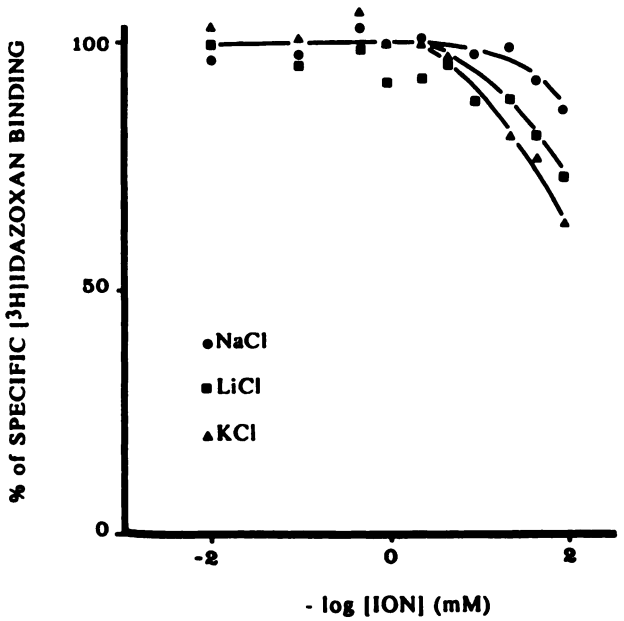


Fig. 5. Effects of NaCl (●), LiCl (■), and KCl (△) on [³H]idazoxan binding. Studies were performed in the presence of 11 nM [³H]idazoxan. In order to prevent binding of [³H]idazoxan to α_2-adrenoceptors, [³H]idazoxan binding studies were performed in the presence of 10^{-6} M yohimbine. The data (means of three experiments) are expressed as a percentage of specific [³H]idazoxan binding in the absence of ions (Tris buffer).

37 ± 5% for KCl, 27 ± 4% for LiCl, and 13 ± 2% for NaCl (four experiments). No effect on nonspecific binding was observed. In order to determine the effect of monovalent ions, comparative saturation studies were performed with [³H]idazoxan and [³H]RX821002 in the presence or absence of 100 mM KCl (Fig. 6). B_{max} values were not statistically different but the K_D of [³H]idazoxan binding at NAIBS was significantly ($p < 0.001$) increased in the presence of 100 mM KCl (from K_D = 14.3 ± 2.0 nM to K_D = 26.3 ± 1.5 nM, five experiments). Such an increase in the K_D suggested a competitive interaction between KCl and [³H]idazoxan binding sites. In order to better understand the nature of the interaction of KCl with NAIBS, the effect of 100 mM KCl on the dissociation rate of [³H]idazoxan was investigated. The radioligand was incubated with the membranes, in the presence of 10^{-6} M yohimbine, for 45 min. Reversibility of the [³H]idazoxan binding was assessed by adding an excess of unlabeled idazoxan (10^{-5} M) in the presence of buffer alone or with 100 mM KCl. As shown Fig. 7, the dissociation rate constant was increased by 100 mM KCl from 0.17 ± 0.01 to 0.29 ± 0.01 min^{-1} ($p < 0.01$, three experiments), suggesting that the cation acts via an allosteric site and not directly with the NAIBS.

Effect of benextramine and phenoxybenzamine on [³H]idazoxan and [³H]RX821002 binding. The apparent affinity of the two irreversible α-antagonists (benextramine

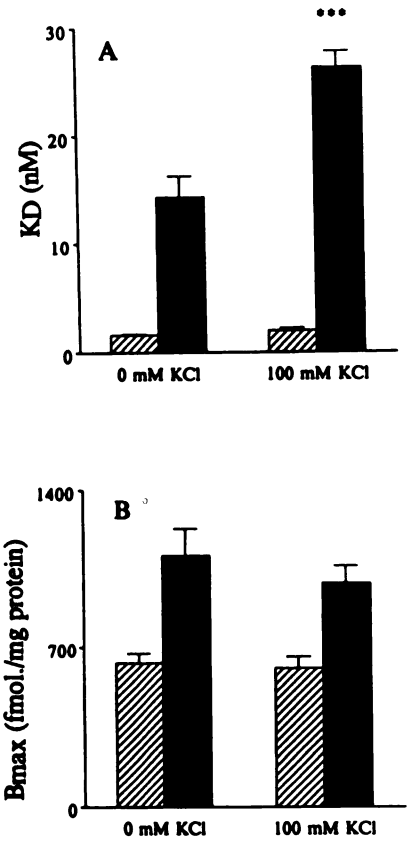


Fig. 6. Binding parameters of [^{3}H]idazoxan (■) and [^{3}H]RX821002 (▨) at NAIBS and α_2-adrenoceptors in the absence or presence of 100 mM KCl. Human fat cell membranes were incubated for 45 min at 25° with different concentrations of [^{3}H]idazoxan (0.5 to 30 nM) and [^{3}H]RX821002 (0.2 to 10 nM). In order to prevent binding of [^{3}H]idazoxan to α_2-adrenoceptors, [^{3}H]idazoxan binding studies were performed in the presence of 10^{-6} M yohimbine. The dissociation constant (K_D) (A) and the maximum number of binding sites (B_{max}) (B) were calculated from equilibrium data using computer-assisted linear transformation of the saturation isotherm (13). The values are mean ± standard error from parallel experiments run on five different membrane preparations. *** Significant difference at $p < 0.001$.

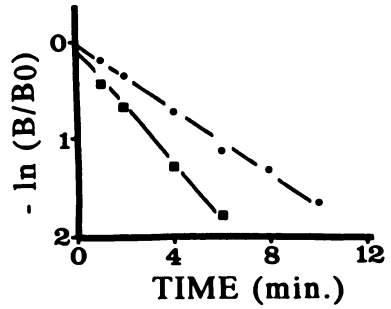


Fig. 7. Effect of 100 mM KCl on the [^{3}H]idazoxan dissociation reaction. A membrane preparation was preincubated for 45 min at 25° in the presence of 12 nM [^{3}H]idazoxan and 10^{-6} M yohimbine. An excess unlabeled idazoxan (10^{-5} M) was added at time zero with (■) or without (●) 100 mM KCl. Specific binding was determined in triplicate at the time indicated. The data from this experiment are representative of those obtained in two others. In this representation, B_0 is the amount of [^{3}H]idazoxan bound at steady state and B corresponds to the binding of [^{3}H]idazoxan at the time indicated. The slope represents the dissociation rate constant (k_{-1}).

and phenoxybenzamine) for human fat cell α_2-adrenoceptors and NAIBS was assessed by determining their potency to inhibit [^{3}H]idazoxan and [^{3}H]RX821002 binding (Fig. 8). Benextramine inhibited [^{3}H]RX821002 and [^{3}H]idazoxan binding (respective EC_{50} values, 303 ± 41 and 5329 ± 21 nM) more potently than did phenoxybenzamine (respective EC_{50} values, 1066 ± 70 and 88700 ± 18800 nM).

Treatment of human fat cell membranes with both irreversible α-blockers was performed as described in Materials and Methods. If one postulates that they bind irreversibly at the α_2-adrenoceptor, pretreatment of the membrane with 10^{-5} M phenoxybenzamine should block 90% of the α_2-adrenoceptors without alteration of the NAIBS, if these sites are not related to α_2-adrenoceptors. On the other hand, a 5×10^{-5} M concentration of benextramine was chosen to block both the α_2-adrenoceptors and the NAIBS (Fig. 8). The results from this study are depicted in Fig. 9. Whichever treatments were performed, the K_D values were not significantly different (not shown). Phenoxybenzamine produced a 90% reduction in the number of [^{3}H]RX821002 binding sites but did not change the number of NAIBS. The blocking effect of 10^{-5} M phenoxybenzamine was totally prevented by the prior addition of 10^{-4} M yohimbine to the incubation medium (not shown). Treatment with 5×10^{-5} M benextramine blocked the α_2-adrenoceptor but, suprisingly, did not impair the [^{3}H]idazoxan binding, indicating that this α-antagonist is not an irreversible ligand on NAIBS.

Discussion

The aim of the present study was to investigate the binding properties of two related radioligands, [^{3}H]idazoxan and [^{3}H]RX821002, in human adipose tissue. Idazoxan is an α_2-adrenergic antagonist that has a higher α_2/α_1-selectivity ratio than yohimbine (22) and, until recently, its tritiated form was considered to be one of the most specific α_2-adrenergic radioligands

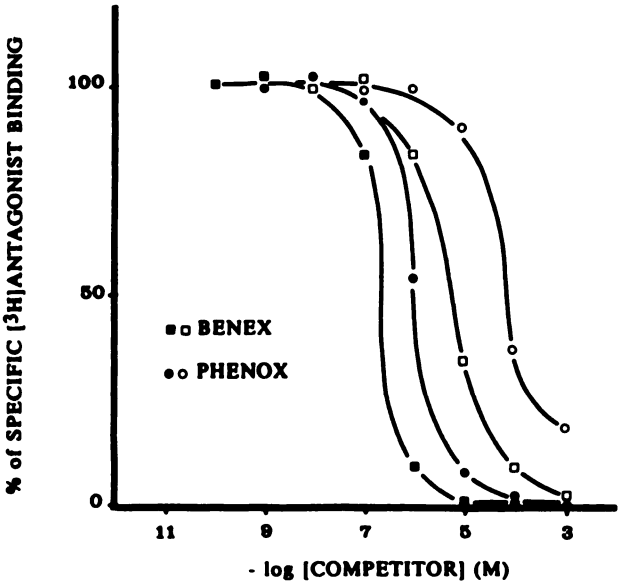


Fig. 8. Inhibition of [^{3}H]idazoxan (□, ○) and [^{3}H]RX821002 (■, ●) binding by benextramine (■, □) and phenoxybenzamine (●, ○). Inhibition studies were performed in the presence of 11–12 nM [^{3}H]idazoxan or 1–1.5 nM [^{3}H]RX821002. In order to prevent binding of [^{3}H]idazoxan to α_2-adrenoceptors, [^{3}H]idazoxan binding studies were performed in the presence of 10^{-6} M yohimbine. The data are expressed as a percentage of specific [^{3}H]radioligand bound.

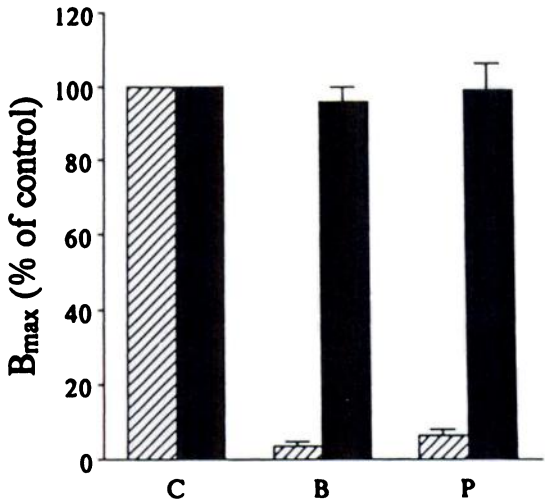


Fig. 9. Comparative binding of [^{3}H]idazoxan (■) and [^{3}H]RX821002 (▨) at NAIBS and α_2-adrenoceptors after treatment of the human fat cell membranes with benextramine or phenoxybenzamine. Treatments were performed as described in Materials and Methods. After extensive washing, the human fat cell membranes were incubated for 45 min at 25° with different concentrations of [^{3}H]idazoxan (0.5 to 30 nM) and [^{3}H] RX821002 (0.2 to 10 nM). In order to prevent binding of [^{3}H]idazoxan to α_2-adrenoceptors, [^{3}H]idazoxan binding studies were performed in the presence of 10^{-6} M yohimbine. The dissociation constant (K_D) and the maximum number of binding sites (B_{max}) were calculated from equilibrium data using computer-assisted linear transformation of the saturation isotherm (13). Whichever treatments were performed, the K_D values were not significantly changed. B_{max} values in treated membranes are expressed as a percentage of control (membranes treated with buffer alone). Values are means ± standard errors of three experiments. *C*, Untreated membranes; *B*, 5×10^{-5} M benextramine-treated membranes; *P*, 10^{-5} M phenoxybenzamine-treated membranes.

(5). Its 2-methoxy derivative, RX821002, was reported as being a more selective and potent α_2-antagonist than idazoxan (17), and we have previously shown that [^{3}H]RX821002 is a more convenient probe to label α_{2A}-adrenoceptors than [^{3}H]yohimbine or [^{3}H]idazoxan (23). It was, therefore, puzzling to find that [^{3}H]RX821002 and [^{3}H]idazoxan labeled different binding site populations in human fat cells.

This observation was initially based on (+)- and (−)-epinephrine competition curves (Fig. 2). Although, as expected for α_2-adrenoceptors, (−)-epinephrine is always more potent than (+)-epinephrine at [^{3}H]RX821002 binding sites, this is not the case for [^{3}H]idazoxan or [^{3}H]UK14304, suggesting that these two radioligands label α_2-adrenoceptors and additional nonadrenergic sites. These data confirmed the high α_2-selectivity of [^{3}H]RX821002 and its potential utility as a probe for α_2-adrenoceptor studies. Using (+)- and (−)-epinephrine, Convents *et al.* (24, 25) showed, in human and rabbit brain, that [^{3}H]rauwolscine labeled α_2-adrenoceptors and 5-hydroxytryptamine$_{1A}$ receptors and that [^{3}H]idazoxan labeled α_2-adrenoceptors and nonstereoselective binding sites.

The existence of NAIBS has been demonstrated in different rabbit tissues (6–9). Recently, [^{3}H]idazoxan was also reported to label NAIBS in rat and human kidney but it was suggested that these sites were different from the rabbit NAIBS (10). In all these tissues, NAIBS have no affinity for catecholamines and do not appear to be receptors for any known hormone. The pharmacological properties of NAIBS from rabbit tissues present, however, some differences from those from other species. For example, phentolamine, clonidine, and amiloride exhibited a higher affinity for rabbit than for human adipocyte NAIBS. In contrast, UK14304 displayed a higher affinity for human adipocyte NAIBS than for those of the rabbit. In both tissues, however, competition studies performed with imidazoline derivatives clearly showed that the rank order of potency of these compounds was strikingly different for α_2-adrenoceptors and for NAIBS (9) (Table 2). Win52401 was 63 times more potent in displacing [^{3}H]idazoxan binding than [^{3}H]RX821002 binding, whereas 2-substituted imidazoline derivatives like RX821002 and efaroxan lost their ability to interact with NAIBS. Interestingly, an identical structure-activity relationship was found in rabbit adipose tissue.[1] Finally, compounds belonging to other chemical families are also able to interact with NAIBS. Guanidine derivatives interact with both α_2-adrenoceptors and NAIBS with a similar rank order of potency, guanabenz > guanoxan > guanethidine, which is also in agreement with that found in pig kidney (26). Benzazepine derivatives (SKF86466 and SKF104078) are also able to inhibit [^{3}H] idazoxan binding with similar affinities in human adipocyte (Table 3), human kidney (10), and rabbit adipocyte.[1] Thus, it appears that rabbit and human NAIBS share most of their pharmacological properties (i.e., insensitivity to catecholamines, a good affinity for some imidazoline and guanidine derivatives, and the same structure-affinity relationship for idazoxan derivatives) but exhibit some minor differences, which might suggest the existence of different subtypes of NAIBS.

Nonadrenergic binding sites defined with [^{3}H]idazoxan and "imidazol(in)e-preferring sites" defined with [^{3}H]clonidine or *p*-[^{3}H]aminoclonidine have in common their insensitivity to catecholamines and their high affinity for idazoxan and cirazoline. But they appear different through other pharmacological properties. Clonidine, imidazole-4-acetic acid, and cimetidine are potent competitors at imidazol(in)e-preferring sites (27, 28) but, whatever tissues were investigated, they are weak competitors at NAIBS (6, 8, 9) (Tables 2 and 3). In contrast, *p*-[^{3}H] aminoclonidine binding sites are not recognized by guanabenz, which competes for NAIBS with a high affinity (29) (Table 3). These results suggest that, of the various nonadrenergic binding sites labeled by [^{3}H]imidazoline, it is possible to distinguish two types of sites, [^{3}H]clonidine or [^{3}H]aminoclonidine sites defined in the central nervous system and [^{3}H]idazoxan sites, which can probably be divided in at least two subtypes according to the pharmacological data.

Among the different compounds tested, some inhibited [^{3}H] idazoxan binding with pseudo-Hill coefficients different from unity. This was, in particular, the case for the α_2-agonist UK14304. Because agonist binding to α_2-adrenoceptors is sensitive to GTP, we tested the possibility that UK14304 binding was also sensitive to it. The pseudo-Hill coefficient of UK14304 competition curves was increased in the presence of GTP for [^{3}H]RX821002 binding but not for [^{3}H]idazoxan binding. Moreover, addition of GTP to [^{3}H]UK14304 binding medium clearly demonstrated that high affinity state α_2-adrenoceptors labeled by [^{3}H]UK14304, but not additional binding sites, were sensitive to GTP. It is not presently known whether UK14304 is an agonist at NAIBS, but the lack of GTP effects on the parameters of NAIBS-UK14304 interaction indicated that the observed shallow inhibition curve cannot be explained by a mechanism involving a GTP-binding protein.

[1] D. Langin, H. Paris, M. Dauzats, and M. Lafontan, manuscript in preparation.

It was recently hypothesized that NAIBS might be closely related to α_2-adrenoceptors in human tissues, because NAIBS are only present in tissues expressing α_2-adrenoceptors (10). In the present report, saturation analyses were performed to accurately quantify α_2-adrenoceptors and NAIBS in human adipocytes from different anatomical locations. B_{max} values obtained for 19 fat deposits did not indicate a correlation of the numbers of NAIBS and of α_2-adrenoceptors. Moreover, in HT29 cell membranes, which possess α_2-adrenoceptors labeled by [^{3}H]RX821002 and [^{3}H]idazoxan (23), we were unable to demonstrate the existence of NAIBS.[2] These data suggest that α_2-adrenoceptors and NAIBS are independent entities. During the completion of the present work and in agreement with the present results, biochemical studies with solubilized rabbit kidney NAIBS indicated that these sites and α_2-adrenoceptors are distinct molecules (11).

Further evidence for the distinct nature of NAIBS and α_2-adrenoceptors was produced by studies of the effects of benextramine and phenoxybenzamine. These compounds are known to irreversibly block α_2-adrenoceptors by formation of a covalent bond with the target site (30, 31). Because some α-adrenergic drugs have a high affinity for NAIBS, we wondered whether this site could be similarly blocked by irreversible antagonists. First, the apparent affinities of phenoxybenzamine and benextramine were determined. EC_{50} values obtained for [^{3}H]RX821002 binding are similar to those previously reported in HT29 cells (32). As expected from the competition data, pretreatment of the human adipocyte membranes with 10^{-5} M phenoxybenzamine produced a 90% reduction in accessible [^{3}H]RX821002 binding sites but did not change either the K_D or the number of NAIBS (Fig. 9). Alkylation of α_2-adrenoceptors by phenoxybenzamine does not, therefore, modify the binding of [^{3}H]idazoxan, suggesting that NAIBS are not closely linked to the α_2-adrenergic receptor molecule.

On the other hand, treatment with 5×10^{-5} M benextramine, which in competition studies inhibited up to 90% of the binding at NAIBS and α_2-adrenoceptors, irreversibly blocked only α_2-adrenoceptors. Thus, benextramine is a reversible antagonist at NAIBS. This implies that [^{3}H]idazoxan binding sites do not present an exposed target thiol on their surface, as reported for the α_2-adrenoceptors (30), and suggests that the domains of NAIBS and α_2-adrenoceptors involved in the ligand-receptor interactions do not have the same chemical structure. This latter hypothesis was confirmed by studying the effect of ions. Monovalent cations inhibited [^{3}H]idazoxan binding with the following rank order of potency: $K^+ > Li^+ > Na^+$. K^+ provoked a decrease in the affinity of [^{3}H]idazoxan for NAIBS by acting via an allosteric site. The exact nature of the physiological relevance of this interaction remains, however, to be clarified. By contrast, [^{3}H]RX821002 binding at α_2-adrenoceptors was insensitive to cations.

In summary, in human adipose tissue [^{3}H]RX821002 labels only α_2-adrenoceptors, whereas [^{3}H]idazoxan labels α_2-adrenoceptors and nonadrenergic sites. These additional binding sites exhibit pharmacological properties different from those of α_2-adrenoceptors and those of the imidazoline-preferring binding sites described in the central nervous system. The pharmacological delineation of human adipocyte NAIBS suggests that they are slightly different from those found in rabbit adipocytes. Moreover, NAIBS appear to be unrelated to α_2-adrenoceptors and the chemical domains implied in the ligand-receptor interaction seem to be different for the two sites.

[2] D. Langin and H. Paris, unpublished data.

Acknowledgments

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