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[^{3}H]RX821002 (2-methoxyidazoxan) binds to α_2-adrenoceptor subtypes and a non-adrenoceptor imidazoline binding site in rat kidney

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Abstract

The binding of [^{3}H]RX821002 (2-methoxyidazoxan) was evaluated in rat kidney membranes. [^{3}H]RX821002 (0.13–16 nM) recognized a single, saturable binding site with high affinity. Different binding site densities were calculated depending on non-specific binding as defined by (−)-adrenaline or RX821002 (10 μM). Competition assays using (−)-adrenaline and the subtype-selective drugs ARC 239 (2-[2-[4-(*o*-methoxyphenyl)-piperazin-1-yl]-ethyl]-4,4-dimethyl-1,3(2H,4H)-isoquinolindione), BRL 44408 (2-[2H-(1-methyl-1,3-dihydroisoindole)methyl]-4,5-dihydroimidazole), oxymetazoline or prazosin for [^{3}H]RX821002 binding sites revealed the presence of α_{2B}-adrenoceptors (33–51%), α_{2D}-adrenoceptors (15–28%) and an adrenaline-insensitive population (34–40%), sensitive to imidazolines. After the addition of (−)-adrenaline (3 μM) to mask α_2-adrenoceptors, [^{3}H]RX821002 specifically identified a saturable binding site with high affinity ($K_d = 4.9 \pm 1.5$ nM). The pharmacological profile of this non-adrenoceptor, [^{3}H]RX821002 binding site (potencies: efaroxan > clonidine > guanabenz > BRL 44408 > ARC 239 > BU 224 (2-(4,5-dihydroimidaz-2-yl)quinoline) > moxonidine > (−)-noradrenaline > cimetidine) is different to that of imidazoline I_1 or imidazoline I_2 binding sites. Alternative incubation in the presence of ARC 239 (50 nM) to mask α_{2B}-adrenoceptors or BRL 44408 (100 nM) to mask α_{2D}-adrenoceptors confirmed the existence of both α_2-adrenoceptor subtypes and a non-adrenoceptor imidazoline binding site.

Keywords: RX821002; α_2-Adrenoceptors; Imidazoline binding sites; Kidney, rat

1. Introduction

The existence of multiple α_2-adrenoceptor subtypes is based on molecular and pharmacological evidence (Bylund, 1992). In the rat, three genes for α_2-adrenoceptors termed RNG, RG10 and RG20 have been cloned. RNG and RG10 appear to encode the pharmacologically defined α_{2B}- and α_{2C}-adrenoceptor subtypes (Bylund, 1992; Bylund et al., 1992), and it is now widely accepted that the RG20 gene corresponds to the α_{2D}-adrenoceptor subtype, which is the bovine, rat and mouse equivalent of the human and porcine α_{2A}-adrenoceptor subtype (O'Rourke et al., 1994).

One of the biological tissues expressing multiple α_2-adrenoceptor subtypes is the rat kidney (Lorenz et al., 1990; Uhlén and Wikberg, 1991a,b) where the α_{2B}-adrenoceptor subtype constitutes a major component of the total α_2-adrenoceptor population (Connaughton and Docherty, 1990; Uhlén and Wikberg, 1991a,b). In contrast, some functional evidence seems to indicate that the α_{2D}-adrenoceptor subtype is involved in the presynaptic modulation of noradrenaline release in the rat kidney (Schwartz and Malik, 1992; Bohmann et al., 1994).

Previous studies have shown that various adrenergic compounds with an imidazoline/guanidine chemical structure, such as the α_2-adrenoceptor agonists clonidine or guanabenz and the α_2-adrenoceptor antagonist idazoxan, also recognize with high affinity non-adrenoceptor binding sites that have been termed imidazoline sites (for a review see Hamilton, 1992). In rat kidney, at least two different imidazoline sites (termed imidazoline I_1 and imidazoline I_2) (Coupry et al., 1989; Ernsberger et al., 1990, 1993; MacKinnon et al., 1993) and a non-adrenoceptor site for oxazolines (King et al., 1995) have been pharmacologically demonstrated, but their physiological role remains to be elucidated. However, some functional differences between α_2-adrenoceptors and imidazoline sites in the rat kidney have been clearly demonstrated (Bidet et al., 1990; Allan et al., 1993).

The present study was initially designed to evaluate in the same tissue, the pharmacological characteristics of the

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binding of [^{3}H]RX821002 to different α_2-adrenoceptor subtypes as a preliminary step to provide a basis for further functional studies. To quantify the proportion of α_{2B}- and α_{2D}-adrenoceptor subtypes in rat kidney, the methoxy analog of idazoxan, [^{3}H]RX821002, was chosen (Uhlén and Wikberg, 1991a,b). Although this imidazoline radioligand has repeatedly been proposed as a selective α_2-adrenoceptor antagonist lacking activity at imidazoline sites (Langin et al., 1990; Vauquelin et al., 1990; Uhlén and Wikberg, 1991b; Hudson et al., 1992; Miralles et al., 1993; De Vos et al., 1994; Wallace et al., 1994; Erdbrügger et al., 1995), the possibility of labelling non-adrenoceptor imidazoline binding sites with [^{3}H]RX821002 has been reported (Chan et al., 1994). Thus, the hypothetical existence of imidazoline sites identified by [^{3}H]RX821002 in rat kidney was considered and subsequently the pharmacological characteristics of this imidazoline site were evaluated.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (weight 230–260 g) were maintained under a 12:12 h light/dark cycle with free access to food and water. Animal care and all experimental protocols were performed in agreement with European Union regulations. The rats were sacrificed, their kidneys were excised and stored at −70°C until assays were performed.

2.2. Membrane preparation

After thawing, membranes were prepared using established methods (Giralt and García-Sevilla, 1989). Kidneys were homogenized in 5 ml of ice-cold Tris–sucrose buffer (5 mM Tris–HCl; 250 mM sucrose; 1 mM $MgCl_2$; pH 7.4) and centrifuged at $1100 \times g$ for 10 min. The supernatants were then centrifuged at $40\,000 \times g$ for 10 min and the pellets were washed and centrifuged twice with 2 ml of fresh Tris incubation buffer (50 mM Tris–HCl; 1 mM EDTA; 0.1 mM Gpp(NH)p (5′-guanylylimidodiphosphate); 140 mM NaCl; pH 7.5). The final pellet was resuspended in an appropriate volume of incubation buffer and protein levels were measured by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

2.3. Binding assays

The [^{3}H]RX821002 binding assays were performed as described previously (Uhlén and Wikberg, 1991a) by incubating 0.7–1.3 mg protein/ml of the membranes in 550 μl of a solution containing incubation buffer, [^{3}H]RX821002 and different drugs for 30 min at 25°C. In the saturation studies, 0.13 nM to 16 nM of [^{3}H]RX821002 (eight concentrations) were used under standard conditions whereas in competition studies, the membranes were incubated with 2 nM [^{3}H]RX821002 in the absence or presence of various concentrations of drugs (10^{-12}–10^{-3} M; 19 concentrations). Incubations were stopped by diluting the samples with 5 ml of ice-cold Tris incubation buffer (4°C) and then filtered and washed through Whatman GF/C glass fiber filters (Brandel Cell Harvester), which had been presoaked with 0.5% polyethylenimine. Radioactivity on the filters was measured by liquid scintillation spectrometry (Packard 2200CA) in minivials containing 5 ml of OptiPhase 'HiSafe' II cocktail (Wallac, UK).

2.4. Analyses of binding data

Analyses of saturation isotherms (K_d, dissociation constant; B_{max}, maximum density of binding sites) and competition experiments (K_i, inhibition constant) as well as the fitting of data to the appropriate binding model were performed by computer-assisted nonlinear regression using the EBDA-LIGAND programmes (Munson and Rodbard, 1980; McPherson, 1985). Saturation and/or competition curves were simultaneously co-analysed (all experiments together) for the best fit, assuming initially a one-site model of radioligand binding and then assuming two- or three-site binding models (De Lean et al., 1978; Motulsky and Ransnas, 1987). The selection between different binding models was made statistically using the principle of extra sum of squares (F test) and the more complex model was accepted if the resulting p value was less than 0.05. Under these analysis conditions, non-specific binding was evaluated experimentally and was also allowed to be fitted by the LIGAND program (Munson and Rodbard, 1980). Theoretical non-specific binding values are expressed as N which represents the slope of the estimated linear relationship between non-specific binding and the concentration of free radioligand.

Results are expressed as the best fit value ± standard errors (S.E.) as determined by the computer program. These S.E. values obtained from nonlinear regression were not used in further formal statistical calculations.

2.5. Isotopes, drugs and chemicals

[^{3}H]RX821002 (1,4-[6,7(n)-[^{3}H]benzodioxan-2-methoxy-2-yl)-2-imidazoline HCl; specific activity 53–62 Ci/mmol) was purchased from Amersham International (Amersham, UK) and stored at 4°C. For the binding assays, appropriate amounts of the stock solutions were diluted with purified water (Milli-RO, Milli-Q) containing 2.5 mM HCl and 6% ethanol. (−)-Adrenaline bitartrate, amiloride HCl, cimetidine, clonidine HCl, desipramine HCl, Gpp(NH)p (5′-guanylylimidodiphosphate), guanabenz, imidazole-4-acetic acid HCl, (−) noradrenaline HCl, oxymetazoline HCl, prazosin HCl and serotonin HCl were from Sigma (St. Louis, MO, USA); 2-[2-[4-(*o*-

methoxyphenyl)-piperazin-1-yl]-ethyl]-4,4-dimethyl-1,3(2H, 4H)-isoquinolindione HCl (ARC 239) was from K Thomae (Biberach, Germany); 4-fluoro-2-(imidazoline-2-ylamino)-isoindoline maleate (BDF 8933) was from Beiersdorf (Hamburg, Germany); 2-(2-benzofuranyl)-2-imidazoline HCl (2-BFI) and RX821002 HCl were synthesized at S.A. Lasa Laboratorios (Barcelona, Spain); 2-[2H-(1-methyl-1,3-dihydroisoindole)methyl]-4,5-dihydroimidazole (BRL 44408) was from Beecham (Harlow, UK); 2-(4,5-dihydro-imidaz-2-yl)quinoline HCl (BU 224) was from Tocris Cookson (Bristol, UK); cirazoline was from Synthelabo Recherche (Bagneux, France); efaroxan HCl was from Research Biochemicals International (Natick, MA, USA); moxonidine was from Solvay (Hannover, Germany); rilmenidine H_2PO_4 was from Servier (Courbevoie, France). All other chemical reagents were of analytical quality and were purchased from Merck (Darmstadt, Germany) or Sigma.

3. Results

3.1. Characterization of [^{3}H]RX821002 binding sites in rat kidney

In preliminary experiments, the specific binding of [^{3}H]RX821002 to rat kidney membranes was determined in the range 0.13–16 nM. Non-specific binding was fitted by the program. Resulting curves indicated a process of high affinity ($K_d = 1.15 \pm 0.12$ nM) in which a single saturable site with a theoretical B_{max} value of 30 ± 3 fmol/mg protein ($n = 8$) was labelled. The theoretical non-specific binding calculated by the program was $N = 0.003 \pm 0.0003$ which corresponds to specific binding of 89–61%. In order to experimentally determine non-specific binding, [^{3}H]RX821002 binding (2 nM) was determined in the absence, as well as in the presence of similar concentrations (10 μM) of the adrenoceptor compounds (−)-adrenaline, idazoxan, RX821002, BDF 8933 or cirazoline. (−)-Adrenaline and cirazoline displaced 85% and 86% of [^{3}H]RX821002 total binding, whereas RX 821002, BDF 8933 and idazoxan displaced 97%, 98% and 97%, respectively. Because it appeared that [^{3}H]RX821002 binding to kidney membranes could be heterogeneous, non-specific binding was subsequently measured in saturation experiments in the presence of 10 μM (−)-adrenaline (to mask the adrenoceptor component) or in the presence of 10 μM unlabelled RX 821002 (to mask the saturable component). Experimentally evaluated specific binding was a saturable process that indicated a single population of [^{3}H]RX821002 binding sites (Fig. 1). However, the percentage of specific binding was different if the non-specific binding was defined in the presence of 10 μM (−)-adrenaline or in the presence of 10 μM RX821002 (Fig. 1).

All these data suggested the possibility that [^{3}H]RX821002 was able to recognize with high affinity a binding site that was relatively insensitive to the catecholamine (−)-adrenaline. Thus, in order to elucidate the biochemical parameters of this non-adrenoceptor [^{3}H]RX821002 binding site, saturation experiments (0.13–16 nM) were performed in the presence of 3 μM (−)-adrenaline and the non-specific binding was defined as the [^{3}H]RX821002 binding in the presence of 10 μM RX821002. The concentration of (−)-adrenaline (3 μM) was chosen as the most suitable for maximal blockade of adrenoceptor populations without a blockade of non-adrenoceptor binding sites (see below). Under these conditions, [^{3}H]RX821002 labelled a single and saturable site with a K_d value of 4.9 ± 1.5 nM and a B_{max} value of 7 ± 2 fmol/mg protein ($n = 4$) (Fig. 2). This resulted in a proportion of non-adrenoceptor binding sites labelled by [^{3}H]RX821002 in the range of 17% to 47% of total binding sites. Preincubation of the rat kidney membranes

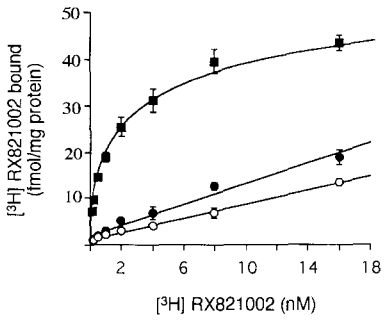

Fig. 1. Saturation curve of [^{3}H]RX821002 binding to rat kidney membranes in the range 0.13–16 nM. Total binding (■), non-specific binding in the presence of 10 μM (−)-adrenaline (●) and non-specific binding in the presence of 10 μM RX821002 (○) are shown. Experimental data were fitted to the equation: $B = \Sigma(B_{max}[L]/(K_d + [L]))$ where B is the concentration of radioligand bound to membranes; B_{max} and K_d are biochemical parameters associated with the specific binding of [^{3}H]RX821002; L is the concentration of free ligand. In both experimental situations, only a single and saturable, high-affinity population could be detected ($K_d = 0.27 \pm 0.05$ nM, $B_{max} = 25 \pm 2$ fmol/mg protein when (−)-adrenaline was used to determine non-specific binding; $K_d = 0.35 \pm 0.06$ nM, $B_{max} = 30 \pm 2$ fmol/mg protein when RX821002 was used to determine non-specific binding). The points represent means ± S.E.M. of 4 separate experiments.

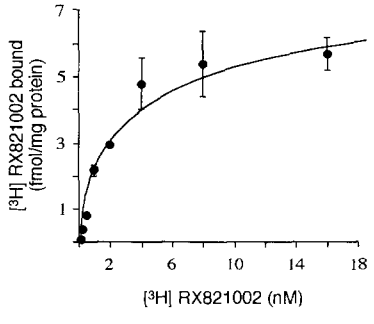

Fig. 2. Saturation curve of the specific binding of [^{3}H]RX821002 (0.13–16 nM) to rat kidney membranes in the presence of 3 μM (−)-adrenaline. Non-specific binding was determined with RX821002 (10 μM) and represented 43-45%. The points are means ± S.E.M. of 4 separate experiments. Other details are as in Fig. 1.

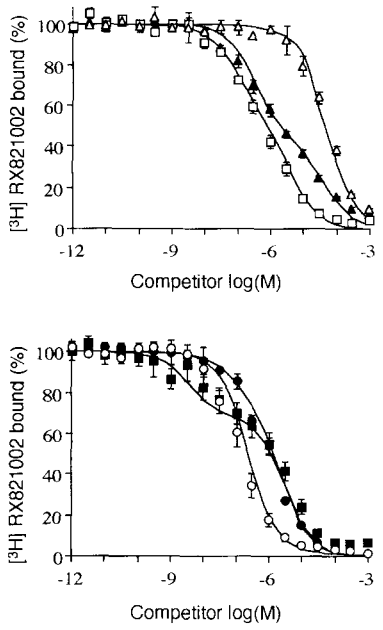

Fig. 3. Competition curves of (−)-adrenaline (▲), serotonin (△), oxymetazoline (□), clonidine (○), ARC 239 (■) and BRL 44408 (●) for [^{3}H]RX821002 binding sites. Rat kidney membranes were incubated at 25°C for 30 min with [^{3}H]RX821002 (2 nM) in the absence or presence of the different drugs (10^{-12}–10^{-3} M; 19 concentrations). Experimental data were fitted to the equation $B = \Sigma(B_{\max i}[L]/K_{di}(1+(I/K_{Ii})))+N[L]$, $i = 1$ to n, where B is the concentration of radioligand bound to membranes; $B_{\max i}$ and K_{di} are the constants for the specific binding of [^{3}H]RX821002 to site i; L is the concentration of free ligand, I is the concentration of the unlabelled displacer; K_{Ii} is the inhibition constant of the displacer for site i and n is the number of sites. For each drug, all competition data (3–6 separate experiments) were simultaneously analyzed assuming initially a one-site model of [^{3}H]RX821002 binding and then assuming two- or three site binding models. The selection between models was made statistically using the extra sum of squares principle (F test). Non-specific binding (N) was allowed to float and fitted by the programme. Note that the competition curves using (−)-adrenaline (▲) ($F[2,71] = 136.88$; $p < 0.001$), oxymetazoline (□) ($F[2,47] = 15.43$; $p < 0.001$), ARC 239 (■) ($F[2,85] = 11.78$; $p < 0.001$) and BRL 444408 (●) ($F[2,47] = 9.73$; $p < 0.001$) are best fitted to a two-site binding model.

for 30 min at 65°C completely abolished [^{3}H]RX821002 specific binding and no specific binding was detected in the absence of kidney membranes. All these data were consistent with the presence of a non-adrenoceptor [^{3}H]RX821002 binding site.

Competition curves using several drugs in the presence of a fixed concentration of [^{3}H]RX821002 (2 nM) were then obtained. Computer-assisted non-linear analyses indicated that clonidine (K_i = 93.5 nM) and guanabenz (K_i = 148 nM) gave rise to monophasic competition curves (Fig. 3). In contrast, competition assays using (−)-adrenaline (K_{iH} = 81.1 nM; K_{iL} = 8.91 μM) and oxymetazoline (K_{iH} = 51.7 nM; K_{iL} = 1.74 μM) resulted in clearly biphasic curves (Fig. 3). Similar results were obtained with the $\alpha_{2A/D}$-adrenoceptor antagonist BRL 44408 (K_{iH} = 17.6 nM; K_{iL} = 0.53 μM) or the $\alpha_{2B/C}$-adrenoceptor antagonists ARC 239 (K_{iH} = 1.13 nM; K_{iL} = 1.01 μM) and prazosin (K_{iH} = 12.4 nM; K_{iL} = 1.92 μM) (Fig. 3). The competition curves obtained for serotonin (K_i = 17.2 μM) and cimetidine (K_i = 50.3 μM) showed a monophasic pattern of low affinity binding. The compound imidazole-4-acetic acid at concentrations of up to 1 mM was inactive at competing against the radioligand binding. The simultaneous analysis of the biphasic competition curves for each drug failed in all cases to yield a three-site binding model of [^{3}H]RX821002. Non-specific binding values estimated by the computer analyses did not differ from those obtained experimentally by 10 μM RX821002 (~3%) and were similar for all the drugs. Therefore, competition experiments seemed to suggest the existence of at least a mixed population of α_2-adrenoceptor subtypes in the rat kidney. However, as can be seen from Fig. 3, a different proportion of binding sites was obtained depending on which drug was considered. The competition curves for (−)-adrenaline demonstrated that the high-affinity component was the predominant binding site (58 ± 3%), with a low-affinity site (42 ± 3%) that could only be identified by higher concentrations of the catecholamine (Fig. 3). The proportion of high-affinity binding sites defined by the $\alpha_{2A/D}$-adrenoceptor selective (relative to $\alpha_{2B/C}$) drug, oxymetazoline (62 ± 12%) was completely different from that obtained by using another $\alpha_{2A/D}$-adrenoceptor selective drug, BRL 44408 (21 ± 7%). Competition assays by the $\alpha_{2B/C}$-adrenoceptor selective (relative to $\alpha_{2A/D}$) drugs, ARC 239 and prazosin indicated that the proportion of high affinity binding sites for these drugs (α_{2B}-adrenoceptors) were 31 ± 6% and 39 ± 5%, respectively. This pattern markedly contrasts with the low-affinity binding site (non α_{2D}-adrenoceptors) observed in BRL 44408 competition studies (79 ± 7% of binding sites) (Fig. 3). The apparent discrepancy in the estimated proportion of α_2-adrenoceptor subtypes evaluated by [^{3}H]RX821002 specific binding (2 nM) could reflect the presence of a third binding site, in consonance with the previous results.

3.2. Discrimination of three [^{3}H]RX821002 binding sites in rat kidney by simultaneous analyses of competition curves

In order to clearly establish the ratio and pharmacological properties of [^{3}H]RX821002 binding sites in the rat kidney, a further analysis was performed. Competition curves using (−)-adrenaline, oxymetazoline, BRL 44408, prazosin and ARC 239 against [^{3}H]RX821002 binding were subjected to simultaneous computer modelling (different drugs being analysed together), testing whether a three-site binding model could best explain the data (i.e. two subtypes of α_2-adrenoceptors and a non-adrenoceptor site relatively insensitive to (−)-adrenaline and recognized by the imidazoline drugs, oxymetazoline and clonidine). Since the non-linear regression analysis (LIGAND pro-

gram) does not generate parameters for more than four different drugs (three cold drugs competing for one radioligand) being analysed together, several separate fits were performed assuming for each set of drugs that one, two or three sites were present and could be identified (Table 1). The model assumed that each individual drug was only able to discriminate between two binding sites, whereas the drugs could recognize the existence of a third [^{3}H]RX821002 binding site when suitably grouped. Thus, K_{i1}, K_{i2} and K_{i3} values could be obtained for each drug representing apparent inhibition constants for α-adrenoceptor, α_{2B}-adrenoceptor and the putative imidazoline binding site, respectively. Using this approach, a significantly better fit to a three-site binding model was demonstrated for [^{3}H]RX821002 in rat kidney (Table 1). The analysis revealed that (−)-adrenaline discriminated between two binding sites, which appeared to be an α_2-adrenoceptor ($\alpha_{2D} + \alpha_{2B}$) component and a catecholamine-insensitive component of the [^{3}H]RX821002 binding. The rank order of K_i values of the adrenergic drugs in competing for the components identified with high affinity by (−)-adrenaline were compatible with α_{2D}- (potencies: oxymetazoline > BRL 44408 > (−)-adrenaline > ARC 239 > prazosin) and α_{2B}- (potencies: ARC 239 > prazosin > (−)-adrenaline > BRL 44408 > oxymetazoline) adrenoceptor subtypes. However, the pharmacological profile of the third binding site (potencies: oxymetazoline > BRL 44408 > ARC 239 > prazosin > (−)-adrenaline) was clearly distinct from that of any α_2-adrenoceptor subtype (Table 1). The analysis also showed that both ARC 239 and prazosin displaced with high affinity the α_{2B}-adrenoceptor subtype component, whereas BRL 44408 displaced with high affinity the α_{2D}-adrenoceptor component (Table 1). In contrast, oxymetazoline displaced with high affinity the α_{2D}-adrenoceptor and non-adrenoceptor components of the curves, the α_{2B}-adrenoceptor component being relatively insensitive to the drug (Table 1). No differences were observed in the analyses of the estimated N values (non-specific binding) in each fit (range 0.00075 to 0.001).

Thus, from the previous results it seemed that the portion of [^{3}H]RX821002 binding displaced by the highest concentrations of (−)-adrenaline (range 3 μM to 1 mM) was recognized with high affinity only by the imidazoline compounds, clonidine and oxymetazoline, and the guanidine compound, guanabenz. Consequently, this adrenaline-insensitive binding site appeared to correspond to a putative population of imidazoline binding sites.

3.3. Discrimination of three [^{3}H]RX821002 binding sites in rat kidney by alternative masking with selective adrenergic drugs

To validate the simultaneous computer modelling and to further determine the pharmacological characteristics of [^{3}H]RX821002 binding to the non-adrenoceptor binding site, competition experiments using several drugs were performed after the inclusion in the assay of 3 μM (−)-adrenaline in order to mask the α_2-adrenoceptor popula-

Table 1
Apparent inhibition constants (K_i) of several drugs in competing for [^{3}H]RX821002 binding sites obtained by simultaneous analysis of competition curves in rat kidney membranes

	K_{i1} (nM)	K_{i2} (nM)	K_{i3} (nM)	Statistical evaluation (2- versus 3-site model)
(−)-Adrenaline	172 ± 58	172 ± 58	19716 ± 3850	
BRL 44408	124 ± 93	641 ± 91	641 ± 9	$F[1,48] = 19.06$
ARC 239	1705 ± 207	2.4 ± 1.6	1705 ± 207	$p < 0.001$
% of sites	27.5 ± 5.5%	32.5 ± 3.8%	40 ± 4.3%	
(−)-Adrenaline	282 ± 123	282 ± 123	30543 ± 9316	
Oxymetazoline	83.6 ± 33.4	1539 ± 372	83.6 ± 33.4	$F[1,50] = 5.64$
ARC 239	2214 ± 409	6.1 ± 4.9	2214 ± 409	$p = 0.021$
% of sites	28.1 ± 8.0%	37.7 ± 5.8%	34.2 ± 5.7%	
(−)-Adrenaline	171 ± 46	171 ± 46	21784 ± 3481	
BRL 44408	41.1 ± 35.6	648 ± 6.4	648 ± 6.4	$F[1,48] = 16.09$
Prazosin	2709 ± 333	14.6 ± 6.7	2709 ± 333	$p < 0.001$
% of sites	20.8 ± 5.2%	40.0 ± 5.7%	39.2 ± 5.6%	
(−)-Adrenaline	260 ± 89	260 ± 89	34917 ± 7912	
Oxymetazoline	39.9 ± 18.8	1176 ± 196	39.9 ± 18.8	$F[1,50] = 6.12$
Prazosin	4692 ± 878	45.4 ± 22.3	4692 ± 878	$p = 0.017$
% of sites	14.9 ± 4.9%	50.9 ± 5.1%	34.2 ± 5.7%	

Analyses were performed with the same competition data for each drug ($n = 3$–6, see text). All the data were simultaneously analyzed (three drugs evaluated together for each group) assuming a one-, two- or three-site binding model of [^{3}H]RX821002 (K_d fixed to 1 nM). The tested model assumed that each individual drug was only able to discriminate between two binding sites, whereas if competing drugs were suitably grouped, three binding sites could be recognized.

Table 2
Inhibition constants (K_i) of several drugs in competing for [^{3}H]RX821002 binding in rat kidney membranes in the presence of 3 μM (−)-adrenaline to mask α_2-adrenoceptors

Drug	K_i (nM)
BDF 8933	1.87 ± 0.3
RX821002	12.5 ± 1.3
Efaroxan	25.5 ± 2.6
Oxymetazoline	48.7 ± 17
Clonidine	172 ± 11
Guanabenz	374 ± 30
BRL 44408	841 ± 639
Rilmenidine	1157 ± 101
ARC 239	1925 ± 185
Prazosin	2431 ± 563
BU 224	7854 ± 708
2-BFI	8974 ± 193
Moxonidine	9310 ± 854
(−)-Noradrenaline	23863 ± 10022
Amiloride	48236 ± 3859
Cimetidine	72326 ± 23144
Imidazole-4-acetic acid	N.D.

Competition data for [^{3}H]RX821002 binding (2 nM) were simultaneously analyzed for each drug (3–5 experiments in each analysis) assuming a one- or more binding site model. K_d for [^{3}H]RX821002 was fixed at 1 nM. Non-specific [^{3}H]RX821002 binding was fitted by the program and was similar for all drugs. K_i values for imidazole-4-acetic could not be determined (N.D.).

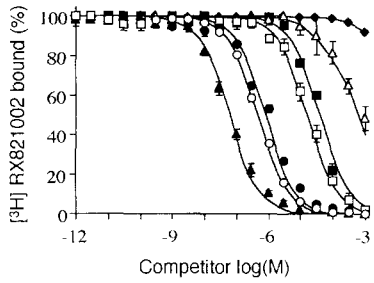

Fig. 4. Competition curves of efaroxan (▲), clonidine (○), guanabenz (●), cimetidine (△), BU 224 (□), moxonidine (■) and imidazole-4-acetic acid (♦) for [^{3}H]RX821002 binding sites in the presence of 3 μM (−)-adrenaline. Rat kidney membranes were incubated at 25°C for 30 min with [^{3}H]RX821002 (2 nM) plus 3 μM (−)-adrenaline in the absence or presence of the different drugs (10^{-12}–10^{-3} M; 19 concentrations). Total binding in the presence of (−)-adrenaline was in the range 1550–2500 dpm and non-specific binding fitted by the programme represented approximately 4% ($N = 0.001$ to 0.00092). Other details are as in Fig. 3.

tion. With this approach, the binding of 2 nM [^{3}H]RX821002 to the α_2-adrenoceptor population was masked by 83%, whereas only 21% of the binding to the population of non-adrenoceptor sites was masked. Since the imidazoline binding sites seem to be heterogeneous and the existence of at least imidazoline I_1 and imidazoline I_2 sites has been assumed (Ernsberger et al., 1992; Michel and Ernsberger, 1992), the pharmacological properties of the [^{3}H]RX821002 binding sites were further characterized by using several drugs with marked selectivity for adrenoceptors or imidazoline sites (Table 2). Under these conditions, all the drugs tested displayed monophasic curves (Fig. 4) and the rank order of K_i values for selected drugs acting on the non-adrenoceptor [^{3}H]RX821002 binding site (potencies: oxymetazoline > BRL 44408 > ARC 239 > prazosin > (−)-noradrenaline) (Table 2) was similar to that obtained through the theoretical analysis assuming a three-site model (K_{i3} values in Table 1). However, al-

Table 3
Inhibition constants (K_i) of several drugs in competing for [^{3}H]RX821002 binding sites in rat kidney membranes in the presence of ARC 239 (50 nM) or BRL 44408 (100 nM) to mask α_{2B}- or α_{2D}-adrenoceptors, respectively

	K_{i1} (nM)	K_{i3} (nM)	Site$_1$ (%)	Site$_3$ (%)
+ ARC 239 (50 nM)				
RX821002	15.1 ± 2.7		100	
(−)-Adrenaline	287 ± 51	19930 ± 2192	30 ± 2	70 ± 2
Oxymetazoline	62.7 ± 12.6		100	
BRL 44408	39.8 ± 23	5244 ± 2799	34 ± 15	66 ± 14
ARC 239	—	—	—	—
Prazosin	1593 ± 191		100	
	K_{i2} (nM)	K_{i3} (nM)	Site$_2$ (%)	Site$_3$ (%)
+ BRL 44408 (100 nM)				
RX821002	9.8 ± 2		100	
(−)-Adrenaline	193 ± 38	15373 ± 2152	62 ± 4	38 ± 3
Oxymetazoline	1751 ± 157	33 ± 11	58 ± 7	42 ± 4
BRL 44408	—	—	—	—
ARC 239	9.3 ± 3.4	1280 ± 358	66 ± 7	34 ± 6
Prazosin	ND	ND	ND	ND

Competition data for [^{3}H]RX821002 (2 nM) were analyzed for each drug (n = 3–6 experiments in each analysis) assuming a one or more binding site model. Selection between models was made statistically using the principle of extra sum of squares (F test). K_d for [^{3}H]RX821002 was fixed to 1 nM. K_{i1}, K_{i2} and K_{i3} represent the inhibition constants for each drug against α_{2D}-adrenoceptors, α_{2B}-adrenoceptors and non-adrenoceptor imidazoline sites, respectively. The proportion for these three [^{3}H]RX821002 binding sites is represented by site$_1$, site$_2$ and site$_3$, respectively. ND, not determined.

though the drugs that recognized the adrenaline-insensitive binding site with high affinity were all imidazoline or guanidine compounds, the pharmacological profile of this [^{3}H]RX821002 binding site (Table 2) did not resemble any of the imidazoline binding sites previously described (Ernsberger et al., 1992; Ernsberger et al., 1993; Miralles et al., 1993; Hudson et al., 1994; Hudson et al., 1995). In this way, the imidazoline I_1 selective drugs clonidine, oxymetazoline and efaroxan recognized the adrenaline-insensitive [^{3}H]RX821002 binding site with high affinity (Table 2). However, cimetidine and imidazole-4-acetic acid (the most imidazoline I_1 selective drugs lacking adrenoceptor activity; see Ernsberger et al., 1992) were very weak or inactive at this binding site (Table 2; Fig. 4). On the other hand, the guanidine compound, guanabenz which possesses a selective high affinity for the imidazoline I_2 site (Ernsberger et al., 1993; Miralles et al., 1993) displayed a competition curve with K_i values in the nanomolar range, whereas the imidazoline I_2 selective drugs BU 224 and 2-BFI showed low affinity for the same binding site (Table 2; Fig. 4). The low affinity binding showed by (−)-noradrenaline for the adrenaline-insensitive site (Table 2) confirmed that this binding site was not an adrenoceptor.

To further demonstrate the validity of the data obtained from the simultaneous computer modelling, additional masking experiments were performed (Table 3). After the inclusion in the assay of 50 nM ARC 239, the binding of 2 nM [^{3}H]RX821002 to the α_{2B}-adrenoceptor subtype was masked by 85%, whereas only 3% of the binding to α_{2D}-adrenoceptors and to the population of imidazoline sites was masked. Under these α_{2B}-adrenoceptor masking conditions, the competition curves of (−)-adrenaline and BRL 44408 were biphasic showing a minor component (30–34%) of high affinity for these drugs and a major one (66–70%) of low affinity (Table 3). K_i values determined from the analysis were similar to K_{i1} and K_{i3} values obtained in the three-site multicurve analyses (Table 1) and seemed to correspond to α_{2D}-adrenoceptors and imidazoline binding sites, respectively. Oxymetazoline (selective $\alpha_{2A/D}$-adrenoceptor and imidazoline drug) displayed monophasic competition curves of high affinity, whereas prazosin (selective $\alpha_{2B/C}$-adrenoceptor and non-imidazoline drug) displayed monophasic competition curves of low affinity (Table 3). K_i values for these drugs were similar to those obtained in the simultaneous multicurve analyses of the three [^{3}H]RX821002 binding sites (Table 1). As expected, RX821002 showed a monophasic competition curve of high-affinity (Table 3).

Conversely, in the presence of 100 nM BRL 44408 to selectively mask α_{2D}-adrenoceptors, the binding of 2 nM [^{3}H]RX821002 to α_{2D}-adrenoceptors was masked by 49%, whereas 12% of the binding to the α_{2B}-adrenoceptors and to the imidazoline site population was masked. Under α_{2D}-adrenoceptor masking conditions, competition curves of (−)-adrenaline, ARC 239 and oxymetazoline were biphasic, whereas the RX821002 curve remained monophasic. The pattern for the competition curves indicated that (−)-adrenaline and ARC 239 recognized a predominant binding site (62–66%) with high affinity that corresponds to the site recognized with low affinity by oxymetazoline (58 ± 7%) (Table 3). K_i values obtained from the analysis were very close to K_{i2} and K_{i3} values obtained from the three-site multicurve analyses (Table 1). The pharmacological profiles of these [^{3}H]RX821002 binding sites in the presence of BRL 44408 seemed to correspond to α_{2B}-adrenoceptors and imidazoline binding sites, respectively.

4. Discussion

The present study demonstrates that in rat kidney membranes [^{3}H]RX821002 is able to recognize two subtypes of α_2-adrenoceptors and a non-adrenoceptor imidazoline binding site.

Simultaneous analysis of several competition and/or saturation curves represents a useful method to estimate accurately binding parameters when two or more sites are present in the tissue (De Lean et al., 1978; Motulsky and Ransnas, 1987). Using this approach, multicurve analyses performed with suitable, subtype selective drugs allow both inhibition constants and the proportion of binding sites identified with high affinity by the radioligand, to be obtained (Uhlén and Wikberg, 1991b; Wikberg-Matsson et al., 1995). In the present study the experimental design was based on the use of three different drugs, each one with high affinity for one or two of the three hypothetical [^{3}H]RX821002 binding sites and on statistical criteria indicating which model (one, two or three binding sites) best fitted the data. Simultaneous analysis of [^{3}H]RX821002 binding properties indicated that the rat kidney appears to possess a mixed population of α_{2B}-adrenoceptors and α_{2D}-adrenoceptors with a predominance of the α_{2B}-adrenoceptor subtype (33–51%) over the α_{2D}-adrenoceptor subtype (15–18%) (Table 1). Additionally, the multicurve approach allowed the identification of a third [^{3}H]RX821002 binding site (34–40%) (Table 1). This finding was confirmed by selective masking of the adrenoceptor populations (Table 3). Thus, competition curves obtained in the presence of (−)-adrenaline (3 μM) contained information concerning [^{3}H]RX821002 binding to a non-adrenoceptor binding site, whereas those obtained in the presence of ARC 239 (50 nM) contained information of binding to α_{2D}-adrenoceptors and to non-adrenoceptor binding sites and those in the presence of BRL 44408 (100 nM) contained information of [^{3}H]RX821002 binding to α_{2B}-adrenoceptors and to non-adrenoceptor binding sites. Concerning the possibility of α_{2C}-adrenoceptors being identified by [^{3}H]RX821002 in rat kidney, molecular (Lorenz et al., 1990) and biochemical studies (Uhlén and Wikberg, 1991b) have provided evidence that α_{2C}-adren-

oceptors are not expressed in this tissue. Thus, the order of affinities and the ratio of potencies for the drugs included in this study confirm that the proposed α_{2B}-adrenoceptor and α_{2D}-adrenoceptor subtypes are both labelled by [^{3}H]RX821002 (Uhlén and Wikberg, 1991a,b) in rat kidney. This finding is consistent with previous studies using [^{3}H]RX821002 (Uhlén and Wikberg, 1991a,b; Erdbrügger et al., 1995) and is seemingly in conflict with studies using [^{3}H]yohimbine or [^{3}H]rauwolscine as radioligands (Cheung et al., 1986; Michel et al., 1989b; Connaughton and Docherty, 1990; Erdbrügger et al., 1995). The lower affinity of yohimbine and rauwolscine for α_{2D}-adrenoceptors (Uhlén and Wikberg, 1991b) and the lower proportion of the α_{2D}-adrenoceptor subtype as compared with α_{2B}-adrenoceptors (Table 1) could explain the failure to identify α_{2D}-adrenoceptors when [^{3}H]yohimbine or [^{3}H]rauwolscine are used in rat kidney (Uhlén and Wikberg, 1991b; Erdbrügger et al., 1995).

In competition studies, a third binding site (34–40%) identified with high affinity by [^{3}H]RX821002 was obtained (Table 1). The ability of the imidazoline/guanidine drugs oxymetazoline, efaroxan, clonidine and guanabenz, but not (−)-adrenaline or (−)-noradrenaline, to recognize this new site with high affinity (Table 2) suggests that it could represent an imidazoline binding site (imidazoline receptor) in rat kidney (Michel et al., 1989a; Coupry et al., 1989; Ernsberger et al., 1990; MacKinnon et al., 1993; King et al., 1995). This binding site does not seem to correspond to any of the α_2-adrenoceptor subtypes described in rat kidney because, in addition to the low affinity displayed by catecholamines, the drugs ARC 239, prazosin and BRL 44408 also showed low affinity for the site (Table 2). The existence of three [^{3}H]RX821002 binding sites does not appear to represent the high- and low-affinity states of the α_2-adrenoceptor subtypes because in the present assay conditions (Gpp(NH)p, NaCl and EDTA included), agonist drugs only recognize one low-affinity state of the receptor (Uhlén and Wikberg, 1991b; Uhlén et al., 1993). In the rat kidney, serotonin displayed a monophasic competition curve with negligible affinity (Fig. 3), indicating that the non-adrenoceptor binding site identified by [^{3}H]RX821002 does not correspond to 5HT receptors labelled by [^{3}H]RX821002 in human brain (Vauquelin et al., 1990). According to previous studies, the radioligand [^{3}H]RX821002 lacks activity at imidazoline receptors (Langin et al., 1990; Vauquelin et al., 1990; Uhlén and Wikberg, 1991b; Hudson et al., 1992; Miralles et al., 1993; De Vos et al., 1994; Wallace et al., 1994; Wikberg-Matsson et al., 1995). The differences between the current results and those of previous authors are mainly represented by the conditions to define non-specific binding and the tissue involved in the assay. In previous studies, (−)-adrenaline (1–10 μM) (Langin et al., 1990; Miralles et al., 1993; De Vos et al., 1994) or phentolamine (10 μM) (Vauquelin et al., 1990; Hudson et al., 1992; Wallace et al., 1994; Erdbrügger et al., 1995; Heal et al., 1995) were used to define non-specific binding. Under these conditions, (−)-adrenaline ascribes the non-adrenoceptor component of [^{3}H]RX821002 binding to the non-specific population. In addition, a similar conclusion is expected for 10 μM phentolamine which shows affinities for renal imidazoline binding sites in the high micromolar range (MacKinnon et al., 1993). In this context, the present study demonstrates a significant difference between the experimental non-specific [^{3}H]RX821002 binding defined either by (−)-adrenaline (10 μM) or by idazoxan, BDF 8933 and RX821002 (10 μM) (Fig. 1). This finding seems to indicate that a non-adrenoceptor binding site is specifically identified by [^{3}H]RX821002 in rat kidney. This idea is consistent with the results of a recent study (King et al., 1995) where a different residual [^{3}H]p-aminoclonidine binding was observed in the presence of (−)-adrenaline or RX821002 in rat kidney. The consistency of the finding is also drawn from the saturation binding data in the presence of (−)-adrenaline (3 μM). Thus, the remaining [^{3}H]RX821002 binding in the presence of the catecholamine was specific, saturable and of high affinity (Fig. 2), and sensitive to high temperatures, indicating the protein nature of the non-adrenoceptor site identified in rat kidney. However, this fact does not satisfactory explain the differences between the present study and those of Wikberg's group (Uhlén and Wikberg, 1991a,b; Uhlén et al., 1993) who employed the imidazoline compound BDF 8933 (1 μM) to define non-specific binding of [^{3}H]RX821002 in rat kidney. Although the affinity of BDF 8933 for imidazoline sites has not been reported, the K_i values obtained in the presence of (−)-adrenaline (Table 2) and the low percentage of non-specific binding suggests that BDF 8933 competes for the [^{3}H]RX821002 non-adrenoceptor binding sites in rat kidney.

In rat kidney membranes, imidazoline I_1 binding sites labelled with [^{3}H]clonidine/[^{3}H]p-aminoclonidine (Ernsberger et al., 1990, 1992) and imidazoline I_2 binding sites labelled with [^{3}H]idazoxan (Michel et al., 1989a; MacKinnon et al., 1993) have been identified. Our data are consistent with previous findings and support the idea that [^{3}H]RX821002 is able to bind imidazoline sites. The profile of several drugs in competing for the non-adrenoceptor component of [^{3}H]RX821002 binding in the rat kidney is not compatible with the involvement of previously defined imidazoline I_1 or imidazoline I_2 binding sites (Table 2; Fig. 4). Since efaroxan, clonidine and oxymetazoline display K_i values in the nanomolar range, the [^{3}H]RX821002 non-adrenoceptor binding site could represent an imidazoline I_1 site (Ernsberger et al., 1987, 1993; Bricca et al., 1989; Piletz and Sletten, 1993). However, the low affinity shown by moxonidine, cimetidine and imidazole-4-acetic acid do not support this hypothesis (Coupry et al., 1989; Ernsberger et al., 1993; Piletz and Sletten, 1993). Similarly the guanidine compound guanabenz, which is considered as an imidazoline I_2 versus imidazoline I_1 receptor selective drug (Ernsberger et al., 1992), displayed a high affin-

ity for the [^{3}H]RX821002 non-adrenoceptor binding site consistent with an imidazoline I_2 site (Coupry et al., 1989; Vigne et al., 1989; Wikberg et al., 1991; Ernsberger et al., 1993; Miralles et al., 1993; Piletz and Sletten, 1993). However, the affinities of 2-BFI, BU 224, clonidine and oxymetazoline are not consistent with this possibility (Vigne et al., 1989; Wikberg et al., 1991; Hudson et al., 1994; Hudson et al., 1995).

Imidazoline compounds with demonstrated selective affinity for imidazoline I_1 versus imidazoline I_2 binding sites such as clonidine, efaroxan and phentolamine (Ernsberger et al., 1992) modulate the activity of ATP-sensitive K^+ channels, thus acting as functional blockers and promoting insulin secretion (Schultz and Hasselblatt, 1989; Chan et al., 1991; Berdeu et al., 1994; Olmos et al., 1994). This physiological role is independent of α_2-adrenoceptor-mediated insulin release (Schultz and Hasselblatt, 1989; Jonas et al., 1992, 1994; Chan, 1993). RX821002 is able to produce similar effects on insulin secretion via a non-adrenoceptor, imidazoline sensitive mechanism (Berdeu et al., 1994; Olmos et al., 1994). This non-adrenoceptor functional effect of RX821002 seems to be mediated by an atypical imidazoline binding site which shows low affinity for idazoxan (Brown et al., 1993). Such data support the idea that [^{3}H]RX821002 is able to recognize imidazoline binding sites. Moreover, a recent study has reported that [^{3}H]RX821002 labels not only α_2-adrenoceptors but also an additional low affinity binding site in the rat insulinoma cell line RINm5F (Chan et al., 1994). [^{3}H]RX821002 binding sites in RINm5F cells display pharmacological characteristics which are distinct from those of previously defined imidazoline I_1 and imidazoline I_2 sites and their physiological effects probably involve the interaction of the radioligand with the ATP-sensitive K^+ channel complex (Berdeu et al., 1994; Chan et al., 1994; Olmos et al., 1994). Thus, the possibility that the [^{3}H]RX821002 imidazoline binding site may represent a novel, third type of imidazoline binding site that is recognized by agents containing either guanidinium or imidazoline structures should be considered (Chan, 1993). Further studies are required to elucidate the physiological role of this binding site in rat kidney and in other tissues.

In conclusion, the radioligand [^{3}H]RX821002 labels α_{2B}-adrenoceptor and α_{2D}-adrenoceptor subtypes as well as an imidazoline/guanidine binding site in rat kidney membranes. The pharmacological profile of the imidazoline binding site identified by [^{3}H]RX821002 is quite different to that previously described for imidazoline I_1 or imidazoline I_2 receptor subtypes.

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