British Journal of Pharmacology (1999) 128, 1215–1222
http://www.stockton-press.co.uk/bjp

Non-adrenergic binding of [^{3}H]atipamezole in rat kidney – regional distribution and comparison to α_2-adrenoceptors

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1 Atipamezole (4-(2-ethyl-2,3-dihydro-1H-inden-2-yl)-1H-imidazole) was first introduced as a potent and specific α_2-adrenoceptor antagonist, but in some tissues [^{3}H]atipamezole identifies an additional population of binding sites, distinct from both classical α_2-adrenoceptors and I_1- and I_2-imidazoline receptors identified with [^{3}H]*para*-aminoclonidine or [^{3}H]idazoxan.

2 In the present study we have characterized [^{3}H]atipamezole binding sites in rat kidney by receptor autoradiography and membrane binding assays and determined whether they are pharmacologically identical with the previously described binding sites for [^{3}H]*para*-aminoclonidine and [^{3}H]idazoxan. [^{3}H]RX821002 and [^{3}H]rauwolscine were used to compare the regional distribution of α_2-adrenoceptors to that of non-adrenergic binding sites of [^{3}H]atipamezole.

3 Comparative autoradiographic experiments demonstrated the differential localisation of [^{3}H]atipamezole, [^{3}H]RX821002 and [^{3}H]rauwolscine binding sites in rat kidney. The pattern of distribution of non-adrenergic [^{3}H]atipamezole binding sites is clearly distinct from that of α_2-adrenoceptors.

4 The non-adrenergic binding of [^{3}H]atipamezole in rat kidney does not fall into any of the previously identified three classes of imidazoline receptors studied with [^{3}H]*para*-aminoclonidine, [^{3}H]idazoxan and [^{3}H]RX821002.

5 Atipamezole had no inhibitory effect on MAO-A or MAO-B activity in renal membranes, which speaks against the involvement of MAOs in the observed radioligand binding.

Keywords: α_2-adrenoceptors; non-adrenergic binding; imidazoline receptors; rat kidney; [^{3}H]atipamezole; [^{3}H]RX821002; [^{3}H]rauwolscine; receptor autoradiography

Abbreviations: I_{max}, maximal inhibition of specific binding; MPV-295, 4-[2-(2,6-dimethylphenyl)ethyl]imidazole; MPV-624 1-[2-(2,6-dimethylphenyl)ethyl]imidazole; MPV-709B 2-[2-(2,6-dimethylphenyl)ethyl]imidazole; MPV-709D 2-[2-(2,6-dimethylphenyl)ethyl]imidazoline; 8-OH-DPAT, 8-hydroxy-2-[di-*n*-propylamino]tetralin

Introduction

Idazoxan and many other imidazoline and guanidinium derivatives with high binding affinity to α_2-adrenoceptors bind also to non-adrenoceptor binding sites in renal tissues in a variety of species (Coupry *et al*., 1987; Michel *et al*., 1989; Vigne *et al*., 1989; Lachaud-Pettiti *et al*., 1991; Wikberg *et al*., 1992; Evans & Haynes, 1994). These non-adrenergic imidazoline binding sites or I-imidazoline receptors have been subclassified into two major groups (Michel & Insel, 1989; Michel & Ernsberger, 1992; Parini *et al*., 1996). The binding sites identified by [^{3}H]*para*-aminoclonidine, which have high affinity for imidazoline and oxazoline derivatives such as rilmenidine and moxonidine, are designated as I_1-imidazoline receptors, and those identified by [^{3}H]idazoxan, which have high affinity for guanidinium derivatives such as guanabenz, as I_2-imidazoline receptors. It has been hypothesized that a subpopulation of I_2-imidazoline receptors represents a previously unknown ligand binding domain on monoamine oxidase (MAO) which is distinct from MAO's catalytic domain (Raddatz *et al*., 1995). The imidazoline binding site present on pancreatic islet cells and involved in the control of insulin secretion represents an atypical I-imidazoline receptor (Chan *et al*., 1994; Olmos *et al*., 1994); this site has been termed an I_3-imidazoline receptor (Eglen *et al*., 1998).

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The imidazole derivative atipamezole was first described as a potent, specific and selective α_2-adrenoceptor antagonist (Scheinin *et al*., 1988; Virtanen *et al*., 1989). However, in some tissues [^{3}H]atipamezole identifies an additional population of binding sites, distinct from both classic α_2-adrenoceptors and I_1- and I_2-imidazoline receptors identified with [^{3}H]*para*-aminoclonidine or [^{3}H]idazoxan (Sjöholm *et al*., 1992; 1995).

The I_1-imidazoline receptors in the central nervous system and in the kidney may have a role in mediating the hypotensive actions of imidazoline and oxazoline drugs, such as clonidine, rilmenidine and moxonidine (Ernsberger *et al*., 1993). Stimulation of renal I_1-imidazoline receptors with moxonidine increases solute excretion (Allan *et al*., 1993). The role of the I_2-imidazoline receptor in kidney function is more unclear, but it has been suggested that idazoxan inhibits Na^+/H^+ exchange in renal proximal tubule cells through an interaction with I_2-imidazoline receptors (Bidet *et al*., 1990). The purpose of the present study was to characterize [^{3}H]atipamezole binding sites in rat kidney and to determine whether they are pharmacologically identical with the previously described imidazoline receptors identified with [^{3}H]*para*-aminoclonidine and [^{3}H]idazoxan (Ernsberger *et al*., 1990; MacKinnon *et al*., 1993). Receptor autoradiography was complemented with binding assays with membranes of rat renal cortex and outer stripe of the outer medulla for further pharmacological characterization of the [^{3}H]atipamezole binding. [^{3}H]RX821002 and [^{3}H]rauwolscine were used to compare the regional distribution of α_2-adrenoceptors to that of non-adrenergic binding sites of

[^{3}H]atipamezole. Preliminary results of this work have been published in abstract form (Sjöholm *et al*., 1996).

Methods

Tissue preparation

For receptor autoradiography, adult male Sprague-Dawley rats were killed by decapitation and their kidneys were rapidly removed and decapsulated. The kidneys were frozen by immersion in cold isopentane in a dry ice bath. Frozen 14 μm transverse sections were cut and thaw-mounted onto gelatinized slides and stored at -70°C until used for autoradiography. Additional sections were stained with hematoxylin and eosin for histological examination.

For membrane binding assays, kidneys were rapidly removed from decapitated rats (Sprague-Dawley of either sex) and decapsulated followed by dissection. Two regions were prepared from separate kidneys: the renal cortex and the outer stripe of the outer medulla, and the inner medulla. The tissues were homogenized using an Ultra-Turrax (2×15 s) in 20 volumes of ice-cold 50 mM Tris-HCl, 0.8 mM EDTA, pH 7.5 at 4°C. The homogenates were first centrifuged at $1000 \times g$ for 5 min at 4°C to remove large particles, and the supernatants were centrifuged at $45{,}000 \times g$ for 25 min at 4°C. The pellets were suspended in 20 volumes of the same buffer and the homogenization and centrifugation were repeated. The resulting pellets were washed with incubation buffer (50 mM K^+-phosphate buffer, pH 7.4 at 25°C) and recentrifuged at $45{,}000 \times g$ for 25 min at 4°C. The final membrane pellets were diluted with incubation buffer ten times the original wet tissue weight and stored at -70°C.

Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as the reference standard.

Receptor autoradiography

At least three kidneys from three different animals were used in each experiment. The sections were incubated for 60 min ([^{3}H]atipamezole and [^{3}H]rauwolscine) or for 20 min ([^{3}H]RX821002) at room temperature in 50 mM K^+-phosphate buffer (pH 7.4) containing 0.125–16 nM [^{3}H]ligands. Non-specific binding was determined with 10 μM atipamezole ([^{3}H]atipamezole), or 100 μM (−)-adrenaline ([^{3}H]RX821002 and [^{3}H]rauwolscine). In some experiments with [^{3}H]atipamezole, the α_2-adrenoceptor antagonist RX821002 (10 μM) was used to preclude binding to α_2-adrenoceptors. Competition experiments were performed with [^{3}H]ligands (1 nM [^{3}H]atipamezole or 2 nM [^{3}H]RX821002) in the presence of 6 or 7 concentrations of the competitors. Assays were terminated by 2×5 min ([^{3}H]atipamezole), $2 + 3$ min ([^{3}H]RX821002) or 2×30 min ([^{3}H]rauwolscine) rinses in ice-cold assay buffer followed by a dip into ice-cold distilled water to remove salts. These incubation and washing conditions were found to be optimal in preliminary studies with slide-mounted tissue sections.

Radiolabelled, dried tissue sections were apposed to tritium-sensitive film (^{3}H-Hyperfilm; Amersham, U.K.) along with autoradiographic [^{3}H]-microscales (Amersham). After exposure for 5 weeks, films were developed manually using Kodak D-19 developer and Unifix fixer and analysed using a video-based computerized image analysis system (MCID, Imaging Research Inc., St. Catharines, Ontario, Canada). Optical densities were converted to nCi of [^{3}H]ligand bound per mg wet tissue equivalent using a standard curve generated from [^{3}H]standards. The following histological regions were identified (Kriz & Bankir, 1988) and measured: cortex, outer stripe of outer medulla, inner stripe of outer medulla, and inner medulla.

Membrane binding assay

Receptor binding was measured by incubating 50 μl (about 10 μg protein per tube for [^{3}H]atipamezole and 250 μg protein per tube for [^{3}H]RX821002) of membrane suspension with [^{3}H]ligands in a total volume of 250 μl of 50 mM K^+-phosphate buffer (pH 7.4) to equilibrium in a 25°C water bath with shaking. Preliminary kinetic studies demonstrated that at this temperature [^{3}H]atipamezole binding reached equilibrium within 10 min, while [^{3}H]RX821002 binding reached equilibrium within 15 min. In both cases stable levels of binding were maintained for at least another 60 min. Incubation times of 20 min for [^{3}H]atipamezole and 30 min for [^{3}H]RX821002 were therefore used in all experiments with membranes. Assays with [^{3}H]atipamezole were performed in the presence of 10 μM RX821002 to preclude binding to α_2-adrenoceptors. The incubations were terminated by dilution with 2 ml of ice-cold TM-buffer (50 mM Tris, 10 mM $MgCl_2$, pH 7.4 at 4°C) and rapid filtration through presoaked fibreglass filters (Whatman GF/B) using a Brandel (Gaithersburg, MD, U.S.A.) cell harvester. The filters were washed twice with 5 ml of the same buffer, and radioactivity remaining on the filters was quantified by liquid scintillation counting. Non-specific binding was defined with parallel incubations containing 1 μM unlabelled atipamezole or 5 μM detomidine for [^{3}H]atipamezole and 100 μM (−)-adrenaline for [^{3}H]RX821002. In saturation binding studies, the final radioligand concentrations ranged from 0.03–6 nM for [^{3}H]atipamezole and from 0.05–9 nM for [^{3}H]RX821002.

Competition binding assays were performed at radioligand concentrations near the K_D of each radioligand. A wide range of different I_1- or I_2-imidazoline receptor discriminating drugs as well as various imidazoles and structurally related derivatives (MPV-compounds) were used in the competition assays with [^{3}H]atipamezole. [^{3}H]Ligands and 12–16 concentrations of competing drugs were incubated with rat kidney membranes as described earlier. Bound and unbound radioactivities were separated by filtration.

Monoamine oxidase A and B assay

Monoamine oxidase A and B activity was assayed radiochemically as described by Keller *et al*. (1987), with minor modifications. 100 μl of rat renal membranes (about 0.1 and 0.4 mg of protein in MAO-A and -B assays, respectively) were preincubated with 0–10 μM unlabelled atipamezole in reaction buffer for 1 min at 37°C before substrate addition. The reaction was carried out in 0.1 M potassium phosphate buffer (pH 7.4, total volume 0.5 ml) using 200 μM [^{14}C]5-hydroxytryptamine as substrate for MAO-A and 10 μM [^{14}C]phenylethylamine as substrate for MAO-B. After incubation for 10 min at 37°C, the reaction was terminated with 2N HCl. The reaction product was extracted with toluene/ethyl acetate (50/50 v v^{-1}) for MAO-A and with heptane for MAO-B, and the radioactivity contained in the organic phase was quantitated by liquid scintillation counting (1219 RackBeta Spectral, Wallac, Turku, Finland). The results are expressed as nmol of reaction product per mg membrane protein per hour.

Data analysis

The Prism regression analysis program (GraphPad Software, San Diego, CA, U.S.A.) was used to calculate the best-fitting values for the parameters of saturation isotherms (K_D and B_{max}) and competition curves (IC_{50}) under an assumption of no co-operativity between sites (Hill slope $\approx$ 1). The saturation data were analysed using one-site models, which failed to account for another population of binding sites for [^{3}H]rauwolscine in the inner regions of the rat kidney sections, with a K_D clearly lower than the highest employed radioligand concentration. Thus, apparent K_D- and B_{max}-values are given for this radioligand, neglecting the presence of mixed binding site population. The competition results were analysed simultaneously for each set of experiments by including all data for a particular competitor in a single curve-fitting procedure. The reported proportions of specific binding of total bound radioactivity refer to graphically estimated values at radioligand concentrations equal to the calculated K_D-value of each individual experiment. The K_D-values are presented as geometric means $\pm$ s.e.mean. The other results are presented as arithmetic means $\pm$ s.e.mean.

Drugs and chemicals

[^{3}H]Atipamezole (custom synthesized by Amersham, Buckinghamshire, U.K. specific activity 83.9 Ci mmol^{-1}) was a gift from Orion Corporation Farmos (Turku, Finland). [^{3}H]RX821002 (specific activity 54.0 and 59.0 Ci mmol^{-1} for two batches), [^{14}C]5-hydroxytryptamine (specific activity 55.0 Ci mmol^{-1}) and 2-phenyl[^{14}C]ethylamine hydrochloride (specific activity 55.8 Ci mmol^{-1}) were purchased from Amersham, [^{3}H]rauwolscine (specific activity 80.4 Ci mmol^{-1}) was from NEN Life Science Products, Boston, MA, U.S.A. Atipamezole (MPV-1248, 4-[2-ethyl-2,3-dihydro-1H-inden-2-yl]-1H-imidazole), detomidine, MPV-295 (4-[2-(2,6-dimethylphenyl)ethyl]imidazole), MPV-624 (1-[2-(2,6-dimethylphenyl)ethyl] imidazole), MPV-709B (2-[2-(2,6-dimethylphenyl)ethyl] imidazole), MPV-709D (2-[2-(2,6-dimethylphenyl)ethyl]imidazoline (for structures, see Sjöholm *et al*., 1995) and idazoxan were synthesized by Orion Corporation Farmos, Turku, Finland. Phentolamine was from Ciba-Geigy (Basel, Switzerland). The following compounds were obtained from Sigma (St. Louis, MO, U.S.A.): (−)-adrenaline, agmatine, amiloride, cimetidine, 8-OH-DPAT (8-hydroxy-2-[di-*n*-propylamino]tetralin), 2-phenylethylamine and serotonin (5-hydroxytryptamine). Cirazoline, guanabenz, ketoconazole, *para*-aminoclonidine and RX821002 were from Research Biochemicals Inc. (Natick, MA, U.S.A.). Other chemicals were of analytical or reagent grade, and were purchased from commercial suppliers.

Results

Autoradiography of [^{3}H]atipamezole, [^{3}H]RX821002 and [^{3}H]rauwolscine

In sections of rat kidney, specific [^{3}H]atipamezole, [^{3}H]RX821002 and [^{3}H]rauwolscine binding was heterogeneously and differentially distributed (Figure 1, Table 1). The binding of [^{3}H]atipamezole to non-adrenergic binding sites in renal cortex and in the outer stripe of the outer medulla was

Table 1 Autoradiographic analysis of rat kidney sections: affinity and regional distribution of [^{3}H]atipamezole, [^{3}H]RX821002 and [^{3}H]rauwolscine binding

Region	*Radioligand*	*App.* K_D (nM)	*App.* B_{max} (fmol mg^{-1} wet tissue)
Cortex	[^{3}H]Atipamezole	2.58 ± 0.23	301 ± 17.2
	[^{3}H]RX821002	1.66 ± 0.09	57.2 ± 3.49
	[^{3}H]Rauwolscine	1.44 ± 0.07	45.7 ± 0.31
Outer stripe	[^{3}H]Atipamezole	1.40 ± 0.04	550 ± 26.7
	[^{3}H]RX821002	1.48 ± 0.21	47.3 ± 4.41
	[^{3}H]Rauwolscine	1.01 ± 0.15	21.1 ± 1.77
Inner stripe	[^{3}H]Atipamezole	n.m.	n.m.
	[^{3}H]RX821002	0.34 ± 0.07	68.1 ± 1.63
	[^{3}H]Rauwolscine	1.08 ± 0.31	11.0 ± 0.91
Inner medulla	[^{3}H]Atipamezole	n.d.	n.d.
	[^{3}H]RX821002	0.24 ± 0.07	84.7 ± 5.91
	[^{3}H]Rauwolscine	3.05 ± 1.63	12.3 ± 1.52

Values are the mean ± s.e.mean of 3–6 experiments. n.m., low levels of specific binding, not measurable; n.d., no detectable specific binding. Note that the apparent (App.) K_D- and B_{max}-values of [^{3}H]rauwolscine for medullary regions are underestimated due to the use of sub-saturating radioligand concentrations. See also Figures 1 and 2.

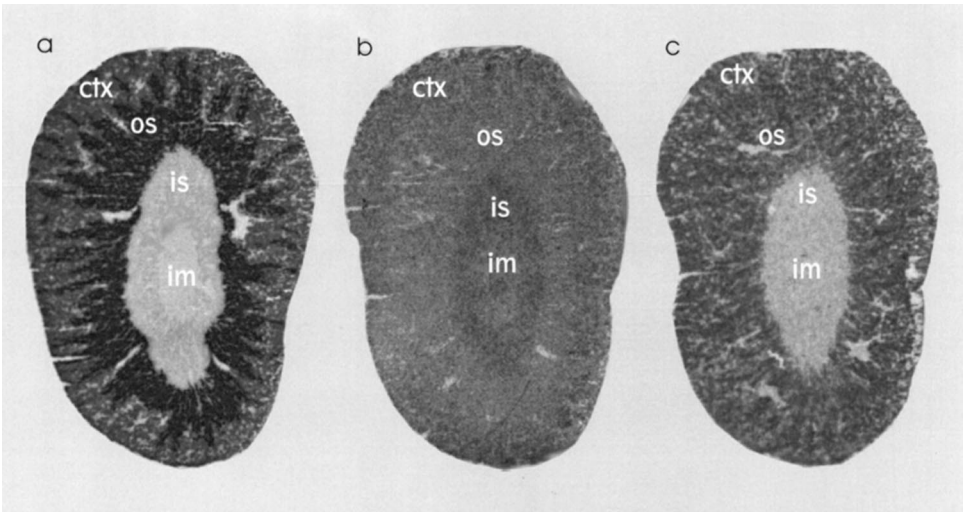

Figure 1 Autoradiographic localization of [^{3}H]atipamezole (8 nM) (a), [^{3}H]RX821002 (16 nM) (b), and [^{3}H]rauwolscine (8 nM) (c) binding sites in rat kidney. See Table 1 for quantitative analysis. Abbreviations: ctx, cortex; os, outer stripe of outer medulla; is, inner stripe of outer medulla; im, inner medulla.

saturable and of high affinity (Table 1, Figure 2a). No specific [^{3}H]atipamezole binding was detected in inner regions of the medulla (Figure 1a). The autoradiographic results obtained with [^{3}H]atipamezole were similar whether 10 μM RX821002 was included in the assays or not. The results of competition binding assays with various drugs, presented as IC_{50} and I_{max} (maximal inhibition of specific binding) are shown in Table 2. In renal cortex, [^{3}H]atipamezole binding was displaced by detomidine with an IC_{50} of 0.18 μM. MPV-624 had intermediate affinity and *para*-aminoclonidine and idazoxan had low affinity. [^{3}H]Atipamezole binding in the outer stripe of the outer medulla appeared to differ from renal cortex: Detomidine and MPV-624 were less potent than in cortex, and idazoxan and *para*-aminoclonidine were unable to displace the radioligand (Figure 3). The putative endogenous imidazoline receptor ligand, agmatine (Li *et al*., 1994), did not displace [^{3}H]atipamezole. RX821002 was also unable to displace [^{3}H]atipamezole binding.

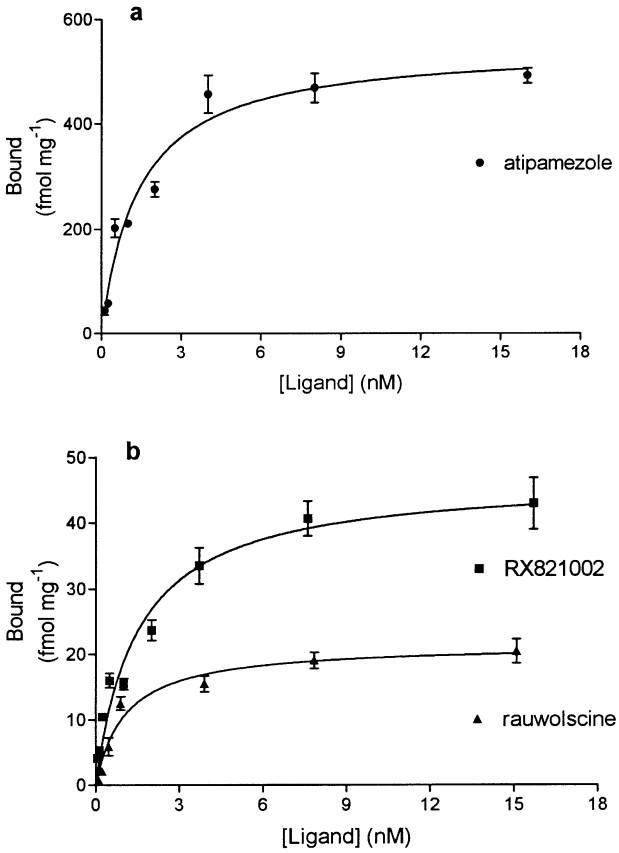

Figure 2 Saturation binding curves of [^{3}H]atipamezole (a), and [^{3}H]RX821002 and [^{3}H]rauwolscine (b) in the outer stripe of the outer medulla of rat kidney sections. Specificity of binding was defined with 10 μM atipamezole in (a) and 100 μM adrenaline in (b). Data points are means$\pm$s.e.mean of 3–6 experiments.

[^{3}H]RX821002 specifically labelled high densities of sites in all regions, with the highest density in the inner medulla (Figures 1b, 2b and Table 1). Competition binding assays were carried out with the subtype-non-selective radioligand [^{3}H]RX821002 and some relatively subtype-selective α_2-adrenergic compounds to determine the α_2-adrenoceptor subtypes present in rat kidney. Prazosin displays relatively low affinity for the $\alpha_{2A/D}$-adrenoceptor subtype and chlorpromazine displays relatively high affinity for the α_{2B}-subtype (Marjamäki *et al*., 1993; O'Rourke *et al*., 1994). The resulting IC_{50}-values for different kidney regions are shown in Table 3.

A high density of specific [^{3}H]rauwolscine binding was detected in the renal cortex, with lower densities in the other regions (Figures 1c, 2b and Table 1).

Non-adrenergic [^{3}H]atipamezole binding to kidney membranes

Saturation studies In the presence of the α_2-adrenoceptor antagonist RX821002 (10 μM) [^{3}H]atipamezole appeared to label a single population of non-adrenergic binding sites with high affinity ($K_D = 0.49 \pm 0.06$ nM) in membranes of rat renal cortex and outer stripe of the outer medulla. The total capacity of specific non-adrenergic [^{3}H]atipamezole binding at equilibrium was 1.84 ± 0.15 pmol per mg membrane protein. At the K_D concentration, the proportion of specific binding of total bound radioligand averaged 88% (range 84–91%, $n = 8$).

Competition studies A series of imidazoles, imidazolines and guanidinium derivatives was tested for their ability to inhibit non-adrenergic binding of [^{3}H]atipamezole (0.6 nM) to rat renal cortex and outer medulla membranes. The specificity of non-adrenergic binding was defined with 5 μM detomidine; the proportion of specific binding ranged from 67–86% ($n = 56$). α_2-Adrenoceptors were blocked by including 10 μM RX821002 in the assays. The non-adrenergic binding of [^{3}H]atipamezole

Table 2 Inhibition of non-adrenergic [^{3}H]atipamezole binding to sections of rat kidney

	Cortex		*Outer stripe of outer medulla*	
Drug	*log IC_{50}* (M)	*I_{max}* (%)	*log IC_{50}* (M)	*I_{max}* (%)
p-Aminoclonidine	-5.59 ± 0.07		> -3.30	29 ± 3
Idazoxan	-4.78 ± 0.12	69 ± 3	> -3.30	15 ± 3
Detomidine	-6.76 ± 0.06		-6.37 ± 0.11	
MPV-624	-5.97 ± 0.06	84 ± 2	-5.24 ± 0.14	73 ± 5
RX821002	> -4	0	> -4	0
Agmatine	> -3	36 ± 2	> -3	0

The sections were incubated with 1 nM [^{3}H]atipamezole in the presence of six or seven concentrations of the competitors. Values are estimates from simultaneous analysis of three experiments$\pm$s.e. of estimate. I_{max} maximal inhibition of specific binding is given if $< 100\%$.

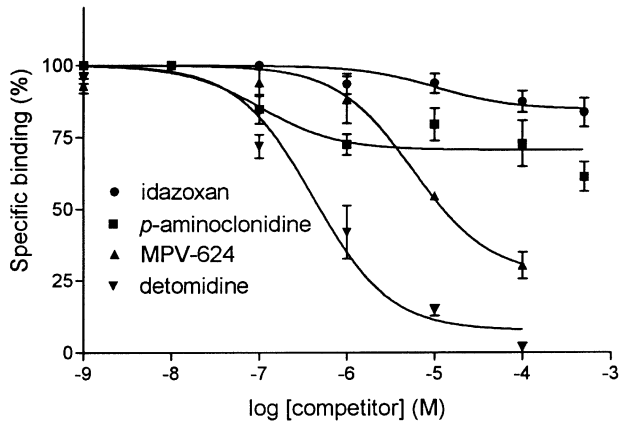

Figure 3 Competition binding curves of 1 nM [^{3}H]atipamezole in the outer stripe of the outer medulla of rat kidney sections. Data points are means$\pm$s.e.mean (shown where greater than the symbols) of three experiments.

Table 3 Inhibition of [^{3}H]RX821002 binding to sections of rat kidney

Drug	*Cortex* *log IC_{50}* (M)	*Outer stripe* *log IC_{50}* (M)	*Inner stripe* *log IC_{50}* (M)	*Inner medulla* *log IC_{50}* (M)
Prazosin	-7.18 ± 0.11	-6.67 ± 0.11	-5.54 ± 0.12	-5.34 ± 0.13
Cholrpromazine	-7.07 ± 0.15	-6.91 ± 0.12	-5.86 ± 0.16	-5.74 ± 0.16
Adrenaline	-6.03 ± 0.08	-5.73 ± 0.08	-5.73 ± 0.08	-5.69 ± 0.07

The sections were incubated with 1 nM [^{3}H]RX821002 in the presence of six or seven concentrations of the competitors. Values are estimates from simultaneous analysis of six experiments ± s.e. of estimate.

Table 4 Inhibition of non-adrenergic [^{3}H]atipamezole binding to membranes of rat kidney cortex and outer stripe of outer medullar in the presence of 10 μM RX821002

Drug	*log $IC_{50(1)}$* (M)	*log $IC_{50(2)}$* (M)
p-Aminoclonidine	-6.15 ± 0.23	-5.00 ± 0.28
Idazoxan	-4.79 ± 0.06	–
Amiloride	-6.17 ± 0.34	-4.56 ± 0.16
Detomidine	-7.82 ± 0.04	–
MPV-295	-8.18 ± 0.07	–
MPV-624	-6.94 ± 0.05	–
MPV-709B	-5.83 ± 0.05	–
MPV-709D	-5.43 ± 0.04	–
Guanabenz	-7.87 ± 0.08	–
Cimetidine	-4.74 ± 0.07	–
Phentolamine	-6.03 ± 0.33	-4.47 ± 0.11
Agmatine	>-3.00	–
Ketoconazole	-6.80 ± 0.06	–

The proportion of the low affinity component ($IC_{50(2)}$) ranged from 54–72%. The membranes were incubated with 0.6 nM [^{3}H]atipamezole in the presence of 12–16 concentrations of the competitors. Values are estimates from simultaneous analysis of 3–5 experiments ± s.e. of estimate. The maximal inhibition of specific binding with agmatine was 36%.

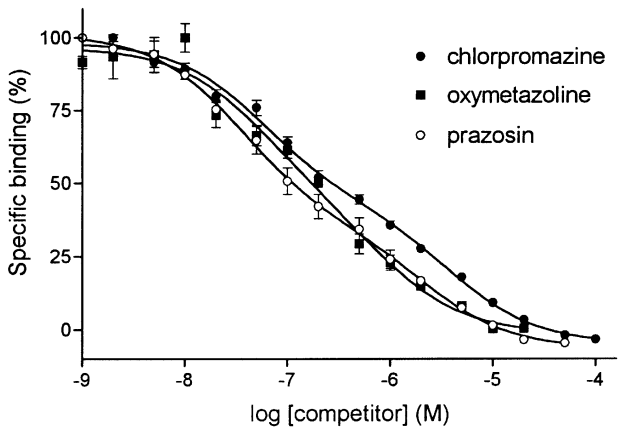

Figure 4 Competition binding curves of 1 nM [^{3}H]RX821002 in membranes of rat renal cortex and the outer stripe of the outer medulla: results for the relatively subtype-selective ligands oxymetazoline, chlorpromazine, and prazosin. Data points represent means ± s.e.mean (shown where greater than the symbols) of five experiments. The data for all three compounds were best described by a two-site model. See text for IC_{50}-values.

was potently displaced by a number of α_2-adrenergic imidazoles, e.g. detomidine and MPV-295 (Table 4). In addition, the cytochrome P450 inhibitor ketoconazole had moderate affinity for these binding sites, while the histamine H_2-antagonist cimetidine had low affinity. Furthermore, imidazoli(di)nes such as *para*-aminoclonidine and phentolamine were moderately, and idazoxan was poorly effective in inhibiting the binding of [^{3}H]atipamezole. The guanidinium derivatives guanabenz and amiloride were also able to inhibit the non-adrenergic binding of [^{3}H]atipamezole. Most of the compounds competed with [^{3}H]atipamezole in a manner consistent with a single population of binding sites but *para*-aminoclonidine, phentolamine and amiloride inhibited [^{3}H]atipamezole binding in a biphasic manner (Table 4).

[^{3}H]RX821002 binding to kidney membranes

In saturation studies, the binding of [^{3}H]RX821002 to membranes from rat renal cortex and outer stripe of the outer medulla was saturable ($B_{max}=120\pm9$ fmol per mg protein) and of high affinity ($K_D=0.96\pm0.06$ nM; $n=6$). Analysis of the competition curves for oxymetazoline, chlorpromazine and prazosin revealed that all binding results with these compounds were significantly better fit to a two-site model of binding than to a one-site model (Figure 4). The calculated IC_{50}-values for the two sites were 52 nM and 0.8 μM for oxymetazoline, 57 nM and 3.8 μM for chlorpromazine and 35 nM and 2.1 μM for prazosin. In each case, the high affinity component of binding represented 50–60% of the specifically bound ligand. In membranes of inner medulla, specific [^{3}H]RX821002 binding was sensitive to the serotonergic compound 8-OH-DPAT, but only at high concentrations of the competitor ($IC_{50}=2.62$ μM). Serotonin itself was not able to inhibit [^{3}H]RX821002 binding.

Monoamine oxidase activity

The basal monoamine oxidase activity in rat renal membranes, in the assay conditions described in the Methods section, was 107 ± 3 nmol mg protein^{-1} h^{-1} ($n=4$) for MAO-A and 12.1 ± 0.6 nmol mg protein^{-1} h^{-1} ($n=3$) for MAO-B. Atipamezole (from 1 nM–10 μM) had no effect on the MAO-A and MAO-B activities (104 ± 0.1 and 11.7 ± 0.3 nmol mg protein^{-1} h^{-1} at 10 μM).

Discussion

We have demonstrated that [^{3}H]atipamezole binding to rat kidney recognizes a large population of non-adrenoceptor sites. The dissociation of [^{3}H]atipamezole from all three α_2-adrenoceptor subtypes is rapid (Halme *et al*., 1995), and no α_2-adrenergic binding was detected in rat kidney sections with [^{3}H]atipamezole because of the extensive washes. Instead, autoradiographic experiments with [^{3}H]RX821002 and [^{3}H]rauwolscine revealed the expected patterns of α_2-adrenergic ligand binding (Stephenson & Summers, 1985; Meister *et al*., 1994).

Non-adrenergic imidazoline binding sites in various tissues are subclassified as I_1-, I_2- and I_3-sites based on their ability to recognize various imidazoline or guanidinium ligands (Michel & Ernsberger, 1992; Eglen *et al*., 1998). The I_1-imidazoline receptor is a [^{3}H]*para*-aminoclonidine binding site found in the

central nervous system and in rat kidney and is recognized by many imidazole compounds (Ernsberger *et al*., 1987). The I_2-imidazoline receptor is an [^{3}H]idazoxan binding site. In contrast to the [^{3}H]*para*-aminoclonidine site described above, I_2-sites have high affinity also for guanidinium derivatives. The I_2-imidazoline receptors can be further subclassified into I_{2A}- and I_{2B}-sites based upon differential recognition of the guanidinium compound amiloride (Michel & Ernsberger, 1992; Parini *et al*., 1996). The I_3-imidazoline receptor is involved in control of insulin secretion. This I_3-binding site is recognized also by [^{3}H]RX821002 (Chan *et al*., 1994). Indeed, the α_2-adrenergic ligand RX821002 (2-methoxy-idazoxan) is an imidazoline compound which functions as an insulin secretagogue in isolated rat islets (Chan *et al*., 1994) and isolated perfused rat pancreas (Berdeu *et al*., 1994); it does not significantly interact with I_1- or I_2-imidazoline receptors (Langin *et al*., 1990) but has relatively high affinity to I_3-imidazoline receptors (K_D minimum estimate 124 nM; Chan *et al*., 1994). Idazoxan is not functionally active in the pancreatic β-cells (Brown *et al*., 1993).

In rat kidney sections, high-affinity [^{3}H]atipamezole binding to renal cortex was displaced with high potency by detomidine. MPV-624 had intermediate affinity and *para*-aminoclonidine had low affinity for these sites. Idazoxan, however, was able to inhibit only 70% of the non-adrenergic binding of [^{3}H]atipamezole demonstrating that the population of [^{3}H]atipamezole binding sites is heterogenous. Each of these cortical [^{3}H]atipamezole binding sites is thus distinctly different from the high-affinity binding sites of [^{3}H]*para*-aminoclonidine and [^{3}H]idazoxan, but the idazoxan-sensitive site could be similar to the low-affinity binding site of [^{3}H]*para*-aminoclonidine in kidney homogenates (MacKinnon *et al*., 1993). [^{3}H]Atipamezole binding in the outer stripe of the outer medulla appeared to differ from that in the renal cortex but it seems to be similar to the non-adrenergic binding of [^{3}H]atipamezole in rat lung (Sjöholm *et al*., 1995). In conclusion, [^{3}H]atipamezole identifies two populations of binding sites in rat kidney sections, both of which are distinctly different from the previously described high-affinity binding sites of [^{3}H]*para*-aminoclonidine and [^{3}H]idazoxan.

A series of competition experiments was performed with membranes from rat renal cortex and outer stripe of outer medulla to further determine the pharmacological specificity of non-adrenergic [^{3}H]atipamezole binding. Cimetidine had low and guanabenz high affinity for non-adrenergic [^{3}H]atipamezole binding sites, which is in contrast to the low-affinity binding sites of [^{3}H]*para*-aminoclonidine (MacKinnon *et al*., 1993), and provides evidence for the identification of these [^{3}H]atipamezole binding sites as a distinct class of high-affinity imidazoline binding sites. Detomidine and the other MPV-compounds confirmed that the non-adrenergic [^{3}H]atipamezole binding has strict requirements for the structure of competing drugs. As shown earlier in neonatal rat lung membranes (Sjöholm *et al*., 1995), 4-imidazoles are more potent than 2-imidazoles in displacing the non-adrenergic binding of [^{3}H]atipamezole.

[^{3}H]Atipamezole is not the only α_2-adrenoceptor ligand which binds to non-adrenergic binding sites with binding characteristics that appear to be distinct from those previously defined for I_1-, I_2-, or I_3-imidazoline receptors. Other such radioligands include [^{3}H]rilmenidine, the oxazoline analogue of clonidine (King *et al*., 1992), the imidazoline [^{3}H]cirazoline (Angel *et al*., 1995) and the imidazoles [^{3}H]mivazerol and [^{3}H]dexmedetomidine (Flamez *et al*., 1997; Savola & Savola, 1996). Rilmenidine has been described as being a high-affinity I_1-imidazoline receptor ligand (Ernsberger *et al*., 1993), but [^{3}H]rilmenidine binding in rat brain and kidney also identifies a non-adrenergic binding site for oxazolines different from I-imidazoline receptors labelled by [^{3}H]*para*-aminoclonidine and [^{3}H]idazoxan (King *et al*., 1995). [^{3}H]Rilmenidine binding has a similar distribution as [^{3}H]atipamezole binding in rat kidney but the total amount of binding is only 50% of that of [^{3}H]atipamezole. Furthermore, unlabelled atipamezole has only micromolar affinity for the non-adrenergic [^{3}H]rilmenidine binding site in membranes from rat cerebral cortex (King *et al*., 1992). [^{3}H]Cirazoline binding does not clearly fall into the I_1- and I_2-type imidazoline binding profiles, and may represent another yet uncharacterized subtype of I-imidazoline receptors (Angel *et al*., 1995; Le Rouzic *et al*., 1995). The non-adrenergic imidazoline binding of [^{3}H]mivazerol and [^{3}H]dexmedetomidine have some similarities to the non-adrenergic binding of [^{3}H]atipamezole (Flamez *et al*., 1997; Savola & Savola, 1996). In human striatum, [^{3}H]mivazerol binding sites display high affinity for dexmedetomidine but low affinity for clonidine and for idazoxan (Flamez *et al*., 1997). The most effective compounds to inhibit non-adrenergic [^{3}H]dexmedetomidine binding to spinal cord membranes of adult rats are atipamezole and other compounds having an imidazole structure (Savola & Savola, 1996). In contrast with the non-adrenergic [^{3}H]atipamezole binding site in rat lung and also with the binding site labelled by [^{3}H]dexmedetomidine (Sjöholm *et al*., 1995; Savola & Savola, 1996), the non-adrenergic [^{3}H]atipamezole binding site in rat kidney has high affinity for guanabenz. Furthermore, *para*-aminoclonidine and amiloride have higher affinity for the non-adrenergic [^{3}H]atipamezole binding site in rat kidney than in rat lung (Sjöholm *et al*., 1995).

Subcellular fractionation of human and rabbit liver has demonstrated that [^{3}H]idazoxan binding to I_2-imidazoline receptors was localized to mitochondrial fractions (Tesson *et al*., 1991). Further purification of mitochondria from rabbit liver revealed that [^{3}H]idazoxan binding was associated with monoamine oxidase (MAO) activity in the outer mitochondrial membranes (Tesson *et al*., 1991). The MAO-A inhibitor clorgyline has high affinity for rat brain I_2-imidazoline receptors. Tissues rich in MAO activity such as placenta, brain, liver and kidney have also high densities of I_2-imidazoline receptors (Weyler & Salach, 1985; Perry *et al*., 1988; Tesson *et al*., 1991; Diamant *et al*., 1992). In recombinant yeast cells expressing human MAO-A or MAO-B, [^{3}H]idazoxan binding is inhibited by the I_2-imidazoline receptor ligands cirazoline and guanabenz (Tesson *et al*., 1995). These results suggest that the I_2-imidazoline receptor may be identical to or closely associated with one or both types of MAO (Parini *et al*., 1996). Atipamezole had no effect on MAO-A or MAO-B activity in renal membranes, which speaks against [^{3}H]atipamezole binding to the catalytic site of MAO or to a distinct regulatory domain of the enzyme protein. On the other hand, ketoconazole, an antifungal agent known to bind to cytochrome P450 enzymes (Halpert *et al*., 1994), was able to inhibit non-adrenergic [^{3}H]atipamezole binding in rat renal membranes. Further studies are necessary to determine whether the non-adrenoceptor site labelled by [^{3}H]atipamezole is associated with the cytochrome P450s.

In situ mRNA hybridization studies in rat kidney show that the predominant α_2-adrenoreceptor subtype expressed in the inner medulla is the $\alpha_{2A/D}$, and the predominant subtype expressed in the outer stripe of the outer medulla radiating into the cortex is the α_{2B}, whereas the α_{2C}-adrenoreceptor probe weakly labelled the inner medulla (Meister *et al*., 1994). The low density and characteristic distribution pattern of [^{3}H]rauwolscine binding in rat kidney sections may be due to

the low affinity of the compound for rat $\alpha_{2A/D}$-adrenoceptors (Harrison *et al*., 1991). In the inner stripe of the outer medulla and in the inner medulla the total amount of [^{3}H]rauwolscine binding was only 15% of [^{3}H]RX821002 binding, probably because of the employed sub-saturating concentration of [^{3}H]rauwolscine. This region has an enrichment of $\alpha_{2A/D}$-adrenoceptor mRNA associated with collecting ducts (Meister *et al*., 1994). The high expression of α_{2B}-adrenoceptor mRNA in proximal tubules in the outer stripe of the outer medulla (Meister *et al*., 1994) correlates with the relative enrichment of [^{3}H]rauwolscine binding in this region in our study. Results from our competition studies with [^{3}H]RX821002 binding at α_2-adrenoceptors in rat kidney sections supplied pharmacological evidence for the α_2-adrenoceptor subtype distribution in this tissue. The low affinity of prazosin and chlorpromazine in the inner stripe of the outer medulla and in the inner medulla implies the existence of $\alpha_{2A/D}$-adrenoceptors. In renal cortex and outer stripe of the outer medulla prazosin and chlorpromazine had high affinity for [^{3}H]RX821002 binding confirming the presence of α_{2B}-adrenoceptors. In membranes of rat renal cortex and the outer stripe of the outer medulla, competition studies with [^{3}H]RX821002 and three relatively subtype-selective α_2-adrenergic compounds also revealed two populations of binding sites. Thus, in membranes of rat renal cortex and the outer stripe of the outer medulla [^{3}H]RX821002 appears to label both $\alpha_{2A/D}$- and α_{2B}-adrenoceptors, which are present in approximately equal amounts.

It has been reported that [^{3}H]RX821002 binds with high affinity to 5-HT$_{1A}$-receptors (Grijalba *et al*., 1996), and that 8-OH-DPAT can act as a competitive α_2-adrenoceptor antagonist with moderate affinity (Crist & Surprenant, 1987). The low affinity of 8-OH-DPAT at [^{3}H]RX821002 sites in membranes of the inner medulla suggests that these sites are not 5-HT$_{1A}$-receptors. Furthermore, serotonin itself was not able to inhibit [^{3}H]RX821002 binding. From these results we conclude that under the experimental conditions of the present study [^{3}H]RX821002 binds exclusively to α_2-adrenoceptors and not to serotonergic receptors in rat kidney. Higher concentrations of [^{3}H]RX821002 have revealed a population of non-adrenergic imidazoline binding sites in some tissues (Chan *et al*., 1994), but this was not investigated in the current study; α_2-adrenergic drugs completely blocked the binding of the employed [^{3}H]RX821002 concentration.

In conclusion, [^{3}H]atipamezole binding recognizes two types of non-adrenergic imidazoline binding sites in rat kidney. These sites are distinct from the previously described populations of I_1-, I_2- and I_3-imidazoline binding sites in rat tissues. Their molecular characteristics remain to be determined.

The authors wish to thank Dr Jarmo S. Salonen from Orion Corporation Orion Pharma for the supply of [^{3}H]atipamezole for these studies, and Ms Ulla Uoti for expert technical assistance. This work was financially supported by grants from the Technology Development Centre of Finland and the Academy of Finland.

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(Received July 30, 1999
Revised August 26, 1999
Accepted September 2, 1999)