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Short communication

Differential characteristics and localisation of [^{3}H]oxazoline and [^{3}H]imidazoline binding sites in rat kidney

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Abstract

Comparative autoradiography revealed that the imidazolines [^{3}H]*p*-aminoclonidine and [^{3}H]idazoxan labelled high densities of α_{2A}-adrenoceptor sites in the inner medulla and inner stripe of the outer medulla of rat kidney. In contrast, the oxazoline [^{3}H]rilmenidine labelled a high density of non-adrenergic sites in the cortex and outer stripe of the outer medulla, a lower density in the inner stripe and inner medulla and a low density of α_{2A}-adrenoceptor sites in inner medulla. The existence of novel, non-adrenergic oxazoline sites of potential functional importance in rat kidney has important implications for the classification of imidazoline receptors.

Keywords: Imidazoline receptor; α_{2A}-Adrenoceptor; α_{2B}-Adrenoceptor; Kidney, rat; [^{3}H]Rilmenidine; [^{3}H]Idazoxan; Autoradiography

1. Introduction

The existence and heterogenous distribution of α_1- and α_2-adrenoceptors in rat kidney have been previously investigated (for review see Calianos and Muntz, 1990). The pharmacological characteristics and localisation of renal α_2-adrenoceptors have been reported in most mammalian species, including rat (Stephenson and Summers, 1985), dog and human, using the alkaloid radioligand, [^{3}H]rauwolscine (Calianos and Muntz, 1990). These earlier studies suggested that in the rat α_2-adrenoceptors were predominantly localised on cortical proximal tubules, with lower levels on distal tubules, blood vessels and glomeruli (Stephenson and Summers, 1985). Recently, [^{3}H]idazoxan, [^{3}H]*p*-aminoclonidine and [^{3}H]methoxyidazoxan (RX821002) have been employed to label α_2-adrenoceptors in brain and various peripheral tissues. Pharmacological and molecular cloning studies have now identified different subtypes of α_2-adrenoceptors – α_{2A}-, α_{2B}-, α_{2C}- and α_{2D}-adrenoceptors (for review see Bylund, 1992). However, homogenate binding studies with [^{3}H]*p*-aminoclonidine and [^{3}H]idazoxan have also reported the labelling of I_1 and I_2 subtypes of imidazoline-preferring receptor binding sites in peripheral tissues (kidney, placenta, liver) and brain of various species (see Brown et al., 1990; Ernsberger et al., 1990, 1993). As part of our research on α_2-adrenoceptor and imidazoline receptor binding sites labelled by the oxazoline, rilmenidine (King et al., 1992), this study investigated the comparative distribution of binding sites labelled by (i) [^{3}H]rilmenidine, (ii) the α_2-adrenoceptor agonist and putative imidazoline I_1 receptor ligand, [^{3}H]*p*-aminoclonidine, and (iii) the α_2-adrenoceptor antagonist and putative imidazoline I_2 receptor ligand, [^{3}H]idazoxan, in sections of rat kidney. A preliminary report of some of these findings has been published in abstract form (King et al., 1994).

2. Materials and methods

2.1. Tissue preparation

Male Wistar-Kyoto rats (250 g) were stunned and killed by decapitation. Kidneys were rapidly excised,

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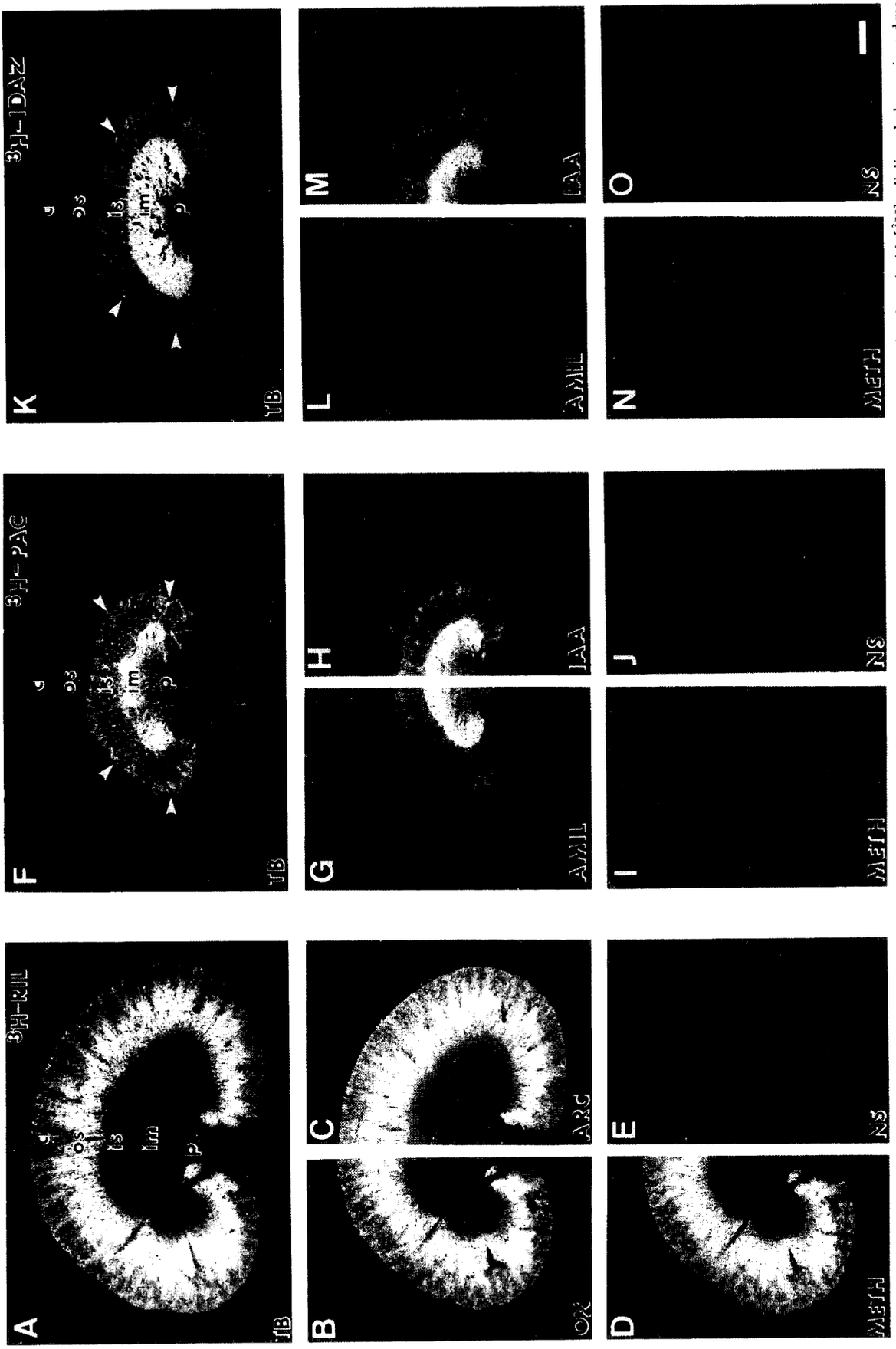

Fig. 1. Autoradiograms of [^{3}H]rilmenidine (RIL), [^{3}H]*p*-aminoclonidine (PAC) and [^{3}H]idazoxan (IDAZ) binding in rat kidney. Sections were incubated with [^{3}H]radioligand plus various drugs to block α_2-adrenoceptors, imidazoline I_1 receptors, or imidazoline I_2 receptors. (A) Total [^{3}H]rilmenidine (40 nM) binding, (B) + 500 nM oxymetazoline (OX), (C) + 500 nM ARC 239 (ARC), (D) + 10 μM methoxyidazoxan (METH), (E) + 10 μM BAY a6781 (non-specific; NS); (F) total [^{3}H]PAC (4 nM) binding, (G) + 100 μM amiloride (AMIL), (H) + 100 μM imidazole-4-acetic acid (IAA), (I) + 10 μM methoxyidazoxan, (J) + 10 μM phentolamine (non-specific); (K) total [^{3}H]IDAZ (5 nM) binding, (L) + 100 μM amiloride, (M) + 100 μM imidazole-4-acetic acid, (N) + 10 μM methoxyidazoxan, (O) + 100 μM cirazoline (non-specific). Note the enrichment of α_2-adrenoceptor sites labelled by [^{3}H]*p*-aminoclonidine and [^{3}H]idazoxan in the inner medulla, in contrast to the intense outer medullary binding of [^{3}H]rilmenidine. Drugs active at α_2-adrenoceptors did not displace [^{3}H]rilmenidine binding from cortex/outer stripe, while these compounds were able to fully displace [^{3}H]*p*-aminoclonidine and [^{3}H]idazoxan binding. Abbreviations: c, cortex; os, outer stripe of outer medulla; is, inner stripe of outer medulla; pap, papilla. Scale bar = 2 mm.

dissected free of the surrounding tissue and renal capsule, then frozen over liquid nitrogen and stored at −70°C. Coronal sections (10 μm) were cut at −17°C, thaw-mounted onto gelatin-coated slides and stored at −70°C for ≤ 2 weeks before use.

2.2. Radioligand binding and autoradiography

Sections were pre-incubated for 15–30 min in buffer (50 mM Tris-HCl pH 7.6, for rilmenidine, 170 mM and 50 mM Tris-HCl pH 7.6, 1 mM EDTA, 10 μM pargyline, for *p*-aminoclonidine and idazoxan, respectively) to remove endogenous ligands and then dried, prior to incubation with a radioligand. Sections were incubated with 40 nM [^{3}H]rilmenidine (38 Ci/mmol, Servier, Paris, France; a concentration $\sim K_D$ for binding in kidney homogenates; unpublished observations) for 60 min at room temperature, in 50 mM Tris-HCl pH 7.6 buffer containing 25 μM EDTA and 10 μM pargyline, in the absence or presence of displacing drugs. Non-specific binding was determined in the presence of the oxazoline 2-[2(*e*)-methyl-6-(*e*)-ethyl-cyclohexyl-1-(*e*)]-amino-2-oxazoline (BAY a6781; 10 μM). Incubations were terminated by washing sections for a total of 4 min. To label α_2-adrenoceptors and/or imidazoline I_1 receptors, sections were incubated with 4–5 nM [^{3}H]*p*-aminoclonidine (56 Ci/mmol, Du Pont-NEN, Boston, MA, USA; a concentration $\sim K_D$ for binding in kidney; Ernsberger et al., 1990) for 60 min at room temperature in 170 mM Tris-HCl, pH 7.6 containing 10 mM $MgCl_2$, 1 mM EDTA and 10 μM pargyline in the absence or presence of displacing drugs. Unbound ligand was removed by washing for 2 × 5 min in ice-cold buffer. Non-specific binding was determined in the presence of 10 μM phentolamine. To label α_2-adrenoceptors and/or imidazoline I_2 receptors, sections were incubated with 4–5 nM [^{3}H]idazoxan (42 Ci/mmol, Amersham International, Amersham, BK, UK; a concentration $\sim K_D$ in kidney; Uhlén and Wikberg, 1991; unpublished observations) for 30 min at room temperature in 50 mM Tris-HCl pH 7.6, containing 10 mM $MgCl_2$, 1 mM EDTA and 10 μM pargyline followed by a 2 min wash in ice-cold buffer. Non-specific binding was determined in the presence of 100 μM cirazoline. All sections were air-dried and desiccated overnight.

Autoradiograms of the slide-mounted sections were prepared by apposition to tritium-sensitive Hyperfilm-^{3}H (Amersham) in X-ray cassettes for 3 ([^{3}H]rilmenidine) and 16 weeks ([^{3}H]*p*-aminoclonidine/idazoxan). [^{3}H]Microscale standards (Amersham) were co-exposed with each film.

2.3. Data analysis

Specific binding was measured using computer-aided densitometry (MCID M1 system, Imaging Research, St. Catharines, OT, Canada) to convert optical densi-

Table 1
Regional distribution and pharmacology of [^{3}H]rilmenidine, [^{3}H]*p*-aminoclonidine and [^{3}H]idazoxan binding to sections of rat kidney

Radioligand	Drug added	Region				
		Cortex	Outer stripe	Inner stripe	Inner medulla	Papilla
[^{3}H]Rilmenidine	Total binding (fmol/mg)	126 ± 8.2	262 ± 28	58 ± 2.7	57 ± 3.3	81 ± 6.1
	Residual specific binding (%) with:					
	1 μM (−)-Adrenaline	89 ± 3	93 ± 2	81 ± 4	73 ± 4	84 ± 2
	10 μM Methoxyidazoxan	90 ± 4	94 ± 3	85 ± 4	75 ± 2	80 ± 2
	100 μM Imidazole-4-acetic acid	102 ± 9	102 ± 6	105 ± 17	107 ± 19	104 ± 17
	100 nM Guanfacine	64 ± 2	59 ± 2	65 ± 4	59 ± 2	71 ± 7
[^{3}H]*p*-Aminoclonidine	Total binding (fmol/mg)	8.9 ± 1.1	6.7 ± 0.4	23 ± 1.5	95 ± 7.6	13 ± 3.0
	Residual specific binding (%) with:					
	1 μM (−)-Adrenaline	10 ± 3	34 ± 5	8 ± 1	2 ± 1	24 ± 5
	10 μM Methoxyidazoxan	6 ± 2	11 ± 5	4 ± 1	1 ± 0	15 ± 5
	100 μM Imidazole-4-acetic acid	93 ± 17	86 ± 12	100 ± 6	96 ± 6	98 ± 30
	100 μM Amiloride	45 ± 4	58 ± 8	74 ± 3	84 ± 7	110 ± 22
[^{3}H]Idazoxan	Total binding (fmol/mg)	13 ± 1.0	11 ± 1.0	24 ± 3.0	77 ± 9.0	15 ± 3.0
	Residual specific binding (%) with:					
	1 μM (−)-Adrenaline	28 ± 6	25 ± 3	8 ± 1	3 ± 1	15 ± 5
	10 μM Methoxyidazoxan	25 ± 2	23 ± 2	6 ± 0	3 ± 1	5 ± 2
	100 μM Imidazole-4-acetic acid	100 ± 7	99 ± 13	97 ± 12	99 ± 12	74 ± 11
	100 μM Amiloride	21 ± 2	25 ± 4	32 ± 4	37 ± 5	36 ± 10

Rat kidney sections were incubated with [^{3}H]radioligands in the presence or absence of drugs, washed, dried and apposed to Hyperfilm-^{3}H for 3 or 16 weeks. Film images were analysed by computer-aided densitometry and optical densities were converted to fmol/mg tissue by interpolation of ^{3}H standard curves. Specific binding values are the mean ± S.E.M. of multiple readings from duplicate, serial sections from 2–6 (rilmenidine) and 4 (*p*-aminoclonidine and idazoxan) rats. Residual specific binding remaining in the presence of drugs is expressed as a percentage. Oxymetazoline (500 nM), idazoxan (10 μM) and cirazoline (1 μM) inhibited [^{3}H]rilmenidine binding by only 0–15% in cortex/outer stripe and by 10–30% in the inner medulla. Cimetidine (100 μM) inhibited [^{3}H]rilmenidine binding by only 5–15% in all regions.

ties from Hyperfilm images to fmol/mg tissue by interpolation of ^3H standard curves and conversion using the specific activity of each radioligand.

2.4. Materials

The following drugs were generously supplied by the companies indicated: amiloride hydrochloride, Merck Sharp and Dohme, Sydney, Australia; 2-(2,4-(*O*-methyoxyphenyl)-piperazan-1-yl)-ethyl-4,4-dimethyl-1,3-(2*H*,4*H*)-isoquinolindione (ARC 239), Karl Thomae, Biberach an der Riss, Germany; BAY a6781, Bayer, Melbourne, Australia; cimetidine, Smith Kline and French Laboratories, Welwyn Gardens City, UK; cirazoline, Synthelabo, Paris, France; guanfacine hydrochloride, Sandoz, Basel, Switzerland; idazoxan, Reckitt and Colman, Hull, UK; oxymetazoline hydrochloride, Allen and Hanburys, Ware, UK; phentolamine hydrochloride, Ciba-Geigy, Basel, Switzerland; rilmenidine, Servier Laboratories, Paris, France. The following drugs were purchased from commercial sources: (−)-adrenaline, imidazole-4-acetic acid and methoxyidazoxan (Sigma Chemical Co., St. Louis, MO, USA).

3. Results

In sections of rat kidney specific [^{3}H]rilmenidine, [^{3}H]*p*-aminoclonidine and [^{3}H]idazoxan binding was heterogeneously and differentially distributed (Fig. 1). High densities of specific [^{3}H]rilmenidine binding (at a concentration $\sim K_D$ for binding to kidney) were present in the cortex and in the outer stripe of the outer medulla, with lower densities in remaining regions (Table 1). In the cortex and outer stripe most specific [^{3}H]rilmenidine binding ($\geq 90\%$) was insensitive to adrenergic drugs, while in the inner stripe and inner medulla these agents inhibited a portion of the specific binding: 1 μM (−)-adrenaline and 10 μM methoxyidazoxan, 15–30% inhibition; 500 nM oxymetazoline, an α_{2A}-adrenoceptor selective drug (Uhlén and Wikberg, 1991) with high affinity for putative imidazoline I_1 receptor sites (Ernsberger et al., 1993) and low affinity for imidazoline I_2 receptors (Michel et al., 1989), 15% inhibition; and 500 nM ARC 239, an α_{2B}-adrenoceptor selective drug (Uhlén and Wikberg, 1991), 0% inhibition (data not shown), suggesting the presence of a population of α_{2A}-adrenoceptors (10–20 fmol/mg) in these areas. Guanfacine (100 nM), an α_{2A}-adrenoceptor selective drug (see Uhlén and Wikberg, 1991) that also has relatively high affinity for [^{3}H]rilmenidine-labelled non-adrenergic sites in brain (King et al., 1992), displaced 35–40% of specific [^{3}H]rilmenidine binding in both cortex/outer stripe and medulla (Fig. 1; Table 1).

In the rat kidney, [^{3}H]*p*-aminoclonidine and [^{3}H]idazoxan (at concentrations $\sim K_D$ for α_2-adrenoceptor and imidazoline receptor binding to rat kidney and brain; Brown et al., 1990; Ernsberger et al., 1990; MacKinnon et al., 1993), specifically labelled a high density of sites in the inner medulla and inner stripe of the outer medulla (Fig. 1; Table 1). Most of this specific [^{3}H]*p*-aminoclonidine and [^{3}H]idazoxan binding ($\geq 90\%$) was displaced by 1 μM (−)-adrenaline or 10 μM methoxyidazoxan. Much lower densities of specific [^{3}H]*p*-aminoclonidine and [^{3}H]idazoxan binding were present in remaining areas of kidney and $\geq 65\%$ was inhibited by (−)-adrenaline and methoxyidazoxan. Thus, in these experiments, little or no specific binding (≤ 2–3 fmol/mg) to imidazoline receptors was detected. Under near-identical conditions, imidazoline I_1 receptor binding sites for [^{3}H]*p*-aminoclonidine have been reported in rat kidney enriched in the medulla based on their inhibition by a putative I_1-selective drug, imidazole-4-acetic acid (Ernsberger et al., 1990), but in the present study, binding of [^{3}H]*p*-aminoclonidine, [^{3}H]idazoxan *and* [^{3}H]rilmenidine was largely unaffected (i.e. inhibited by 0–15%) by a 100 μM concentration of imidazole-4-acetic acid or another imidazole, cimetidine (Table 1). High concentrations (100 μM) of amiloride, a putative imidazoline I_{2A} receptor ligand (see Ernsberger et al., 1993), inhibited 65–85% of specific [^{3}H]idazoxan binding. Amiloride is also known, however, to potently inhibit [^{3}H]idazoxan binding to α_2-adrenoceptors (Howard et al., 1987), an effect consistent with the [^{3}H]idazoxan data presented here.

4. Discussion

The present study demonstrates the differential localisation of high affinity binding sites for radiolabelled oxazoline and imidazoline drugs in the rat kidney. At the nanomolar concentrations of ligand used, [^{3}H]*p*-aminoclonidine and [^{3}H]idazoxan appear to predominantly label only α_2-adrenoceptor sites (i.e. $\geq 90\%$ specific binding is inhibited by (−)-adrenaline and the α_2-adrenoceptor selective antagonist, methoxyidazoxan). The results obtained using [^{3}H]*p*-aminoclonidine are consistent with a recent report that this ligand labels a high density of imidazoline receptor binding sites with only moderate affinity ($K_D \sim 130$ nM) in rat kidney (MacKinnon et al., 1993), but are in contrast to the reported enrichment of imidazoline I_1 receptor binding sites labelled with high affinity (K_D 13 nM) by [^{3}H]*p*-aminoclonidine in the medulla of rat kidney sections (Ernsberger et al., 1990). Our results are also consistent with the findings that *p*-aminoclonidine has very low affinity ($IC_{50} \sim 5$ μM) for non-adrenergic sites labelled by [^{3}H]clonidine in human brainstem ho-

mogenates (Bricca et al., 1994) and that no catecholamine-insensitive binding sites were detected autoradiographically with [^{3}H]*p*-aminoclonidine in human or rat brain (see Bricca et al., 1994; King et al., 1995).

[^{3}H]Idazoxan has been used to label α_2-adrenoceptors and putative imidazoline I_2 receptor binding sites in rat kidney homogenates (Michel et al., 1989; MacKinnon et al., 1993). However, in the present study we saw no clear evidence of a substantial population (i.e. > 2–3 fmol/mg) of high affinity imidazoline I_2 receptor binding in kidney sections. This may due to the differential affinities (K_D values) of the α_2-adrenoceptor and imidazoline I_2 receptor populations present and the resultant differential occupancy, and although both α_2-adrenoceptors and imidazoline I_2 receptors in homogenates of rat brain and kidney reportedly have nanomolar affinity for [^{3}H]idazoxan (Michel et al., 1989; Brown et al., 1990; MacKinnon et al., 1993), it is possible that the use of higher concentrations of [^{3}H]idazoxan (and [^{3}H]*p*-aminoclonidine) and different experimental conditions may label lower affinity imidazoline receptor sites in tissue sections.

The pattern of labelling with [^{3}H]idazoxan was identical to that seen with [^{3}H]*p*-aminoclonidine and quite different to that described using [^{3}H]rauwolscine (Stephenson and Summers, 1985; Calianos and Muntz, 1990). It has been suggested, however, that these imidazoline ligands are selective for α_{2A}-adrenoceptors (Brown et al., 1990; Alburges et al., 1993), while low concentrations of [^{3}H]rauwolscine may selectively label the high and predominant density of α_{2B}-adrenoceptors in rat kidney (i.e. 9 : 1 ratio of α_{2B}- to α_{2A}-adrenoceptors) (Michel et al., 1990; Uhlén and Wikberg, 1991). These results are consistent with the high expression of α_{2A}-adrenoceptor mRNA associated with collecting ducts in the inner medulla, a region enriched in [^{3}H]*p*-aminoclonidine and [^{3}H]idazoxan binding (current study), and the high expression of α_{2B}-adrenoceptor mRNA by proximal tubules in the outer stripe of the outer medulla (Meister et al., 1994), a region enriched in [^{3}H]rauwolscine binding in rat kidney (Stephenson and Summers, 1985).

[^{3}H]Rilmenidine has previously been reported to label a high affinity α_{2A}-adrenoceptor and a lower affinity imidazoline $I_{2\text{-like}}$ receptor binding site in rat brain homogenates and frozen sections (King et al., 1992, 1995). In rat kidney, which contains both α_{2A}- and α_{2B}-adrenoceptors, [^{3}H]rilmenidine detected a small population of inner medullary α_{2A}-adrenoceptors (i.e. inhibited by oxymetazoline, but not ARC239; Uhlén and Wikberg, 1991). In contrast, [^{3}H]rilmenidine labelled a very high density of non-adrenergic binding sites over proximal straight tubules in the outer stripe of the outer medulla and in the cortex (medullary rays) which were inhibited by another oxazoline (Bay a6781) and guanfacine, but not appreciably by the α_2-adrenoceptor/imidazoline I_2 receptor drugs, cirazoline or idazoxan (see Table 1 legend). Rilmenidine has been described as being a high affinity imidazoline I_1 receptor ligand, similar to clonidine and moxonidine, on the basis of their potency against [^{3}H]*p*-aminoclonidine and [^{3}H]clonidine binding to various tissues including rat kidney and common functional effects (see Ernsberger et al., 1993), but our results do not provide evidence to support this in that [^{3}H]rilmenidine binding to kidney is differentially localized to putative imidazoline I_1 receptor sites labelled by [^{3}H]*p*-aminoclonidine and imidazole-4-acetic acid and cimetidine do not inhibit the majority of [^{3}H]rilmenidine binding. Instead, our findings suggest that a unique, high affinity, non-adrenergic binding site population may exist for oxazolines in various mammalian tissues including brain and kidney (King et al., 1992, 1994, 1995) that have tissue-specific differences to imidazoline receptor binding sites labelled by [^{3}H]*p*-aminoclonidine and [^{3}H]idazoxan. Oxazoline radioligands will thus be useful for further investigating the differential characteristics of these renal sites in relation to other putative imidazoline sites and the nature and functional importance of these putative drug receptors in rat and other species including man.

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