0026-895X/89/030324-07$02.00/0

MOLECULAR PHARMACOLOGY, 35: 324-330

[^{3}H]Idazoxan and Some Other α_2-Adrenergic Drugs also Bind with High Affinity to a Nonadrenergic Site

MARTIN C. MICHEL, OTTO-ERICH BRODDE, BERND SCHNEPEL, JOHANNES BEHRENDT, RICHARD TSCHADA, HARVEY J. MOTULSKY, and PAUL A. INSEL

Department of Pharmacology, University of California San Diego, La Jolla, California 92093 (M.C.M., H.J.M., P.A.I.), Departments of Medicine and Urology, University of Essen, Medical School, Essen, West Germany (O.-E.B., B.S., J.B.), and Department of Urology, Klinikum Mannheim, Mannheim, West Germany (R.T.)

Received October 11, 1988; Accepted December 14, 1988

SUMMARY

We compared the pharmacological properties of the α_2-adrenergic radioligand [^{3}H]idazoxan with those of [^{3}H]rauwolscine in rat and [^{3}H]yohimbine in human renal cortical membranes. The density of "specific" [^{3}H]idazoxan binding sites (defined by 100 μM tolazoline) was twice as high as that of [^{3}H]rauwolscine in rat kidney and four times as high as that of [^{3}H]yohimbine in human kidney. A variety of structurally different drugs fully competed for specific [^{3}H]rauwolscine and [^{3}H]yohimbine binding, with affinities appropriate for the interaction with α_2-adrenergic receptors. Specific [^{3}H]idazoxan binding, however, was only partially competed for by the catecholamines epinephrine and norepinephrine in both tissues. Thus, [^{3}H]idazoxan labels both α_2-adrenergic receptors and a nonadrenergic site. Clonidine, B-HT 920, moxonidine, phentolamine, prazosin, yohimbine, dopamine, and serotonin also could not compete for this site. However, UK 14,304, guanabenz, indanidine, tolazoline, oxymetazoline, and SK&F 104,078 competed for the additional [^{3}H]idazoxan sites with affinities similar to those at α_2-adrenergic receptors. [^{3}H]Idazoxan binding substantially in excess of [^{3}H]rauwolscine or [^{3}H]yohimbine binding was also found in human platelets, myometrium, and erythroleukemia (HEL) cells but not in three cell lines lacking α_2-receptors (MDCK, BC3H1, and Jurkat cells). Although we have been unsuccessful thus far in defining the precise nature of the additional [^{3}H]idazoxan binding sites, we hypothesize that these sites may be closely affiliated with α_2-adrenergic receptors but clearly distinct from the catecholamine binding site of the receptor. The results indicate that care must be taken in the use of [^{3}H]idazoxan or drugs that are recognized at its nonadrenergic site when studying α_2-adrenergic effects and receptor subtypes.

Evidence has accumulated that α_2-adrenergic receptors are not homogeneous entities but can be further subdivided into subtypes. Four approaches have been used for defining such subtypes.

1) Bylund and co-workers (1, 2) have found differences in the affinities of various drugs for α_2-adrenergic receptors in different tissues and have proposed a subclassification into α_{2A}- and α_{2B}-adrenergic receptors. In this scheme, oxymetazoline has higher affinity for the α_{2A}- than the α_{2B}-receptor, whereas prazosin and ARC 239 have lower affinity for the α_{2A}- than the α_{2B}-receptor.

2) More than one gene has been found to encode for α_2-adrenergic receptors in humans. One of these genes encodes for the α_2-adrenergic receptors with low affinity for prazosin that is found in human platelets and, thus, may correspond to the α_{2A}-adrenergic receptor (3). Another gene from a human kidney library encodes for an α_2-adrenergic receptor with high affinity for prazosin and, thus, may correspond to the α_{2B}-adrenergic receptor (4).

3) The α_2-adrenergic antagonist SK&F 104,078 has high affinity for postsynaptic α_2-receptors and low affinity for most presynaptic α_2-receptors (5). Inasmuch as SK&F 104,078 has a similar high affinity for both the α_{2A}- and the α_{2B}-adrenergic receptors (4), these data suggest presynaptic α_2-adrenergic receptors may belong to another subtype.

4) Rauwolscine and idazoxan (RX 781094) are considered to be two of the most α_2-selective drugs available (6, 7). However, autoradiographic studies demonstrated that the binding sites identified by [^{3}H]RAU and [^{3}H]IDA in rat brain slices differ in both their pharmacological specificity (8) and their anatomical distribution (9).

In the present study, we set out to assess the possible existence of α_2-adrenergic receptor subtypes in kidney by comparing the pharmacological properties of the binding site for [^{3}H]IDA with those of [^{3}H]RAU in rat and [^{3}H]YOH in human renal

This work was supported by grants from the National Institutes of Health (GM 31987, HL 35846, and HL 35018), the Sandoz Stiftung für Therapeutische Forschung, and the Landesamt für Forschung NRW. M.C.M. is recipient of a fellowship of the Deutsche Forschungsgemeinschaft. H.J.M. is an Established Investigator of the American Heart Association.

ABBREVIATIONS: [^{3}H]RAU, [^{3}H]rauwolscine; [^{3}H]IDA, [^{3}H]idazoxan; [^{3}H]YOH, [^{3}H]yohimbine; MDCK, Madin Darby canine kidney D-1 cells; HEL, human erythroleukemia cells; Gpp(NH)p, guanosine 5′-(β, γ-imido)triphosphate; CDS, clonidine-displacing substance.

cortical membranes. To our surprise, we discovered that [³H]IDA, as well as some other drugs previously used for subclassification of α_2-adrenergic receptors, also binds with high affinity to a novel nonadrenergic site.

Experimental Procedures

Materials. [³H]IDA was purchased from Amersham (Arlington Heights, IL) and [³H]RAU and [³H]YOH from New England Nuclear (Boston, MA); epinephrine, norepinephrine, dopamine, serotonin, tolazoline, oxymetazoline, *p*-NH_2-clonidine, histamine, yohimbine, amiloride, and cimetidine were from Sigma Chemical Co. (St. Louis, MO). The following drugs were gifts of the respective companies: moxonidine (Beiersdorf, Hamburg, FRG), guanabenz (Wyeth, Princeton, NJ), prazosin and UK 14,304 (Pfizer, New York, NY), SK&F 86,466 and SK&F 104,078 (Smith Kline & French, Philadelphia, PA), clonidine (Boehringer Ingelheim, Ingelheim, FRG), phentolamine (Ciba Geigy, Summit, NJ), idazoxan (Reckitt and Colman, Hull, UK), indanidine (Siegfried, Zofingen, Switzerland), cirazoline (Synthelabo, Paris, France), and B-HT 920 (Thomae, Biberach, FRG).

Tissue and cell preparation. Male Sprague-Dawley rats (200–250 g) were obtained from Bantin & Kingman (Palo Alto, CA). After decapitation of the animals, the kidneys were removed and the renal capsule and medulla were dissected and discarded. The remaining renal cortex was weighed, placed into ice-cold preparation buffer (20 mM $NaHCO_3$), minced with scissors, and homogenized with a Tissuemizer for 10 sec at full speed and twice for 20 sec at half speed. The homogenate was centrifuged for 20 min at 40,000 × *g*. The supernatant was discarded and the pellet was resuspended in binding buffer (50 mM Tris-HCl, 0.5 mM EDTA, pH 7.5) to a dilution of 15 ml/g of wet weight and rehomogenized for 10 sec at half speed.

HEL, MDCK, and BC3H1 smooth muscle cells were maintained in tissue culture under previously reported conditions (10–12). Human T (Jurkat) cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. The cells were homogenized and centrifuged using the same method as for rat kidneys.

Human renal cortex was obtained from patients undergoing nephrectomy because of hypernephroma; the tissue used in this study was from macroscopically and histologically tumor-free areas. Membranes were prepared as previously described (13). Human myometrial tissue was obtained from patients undergoing cesarean section. The procedures for membrane preparation and radioligand binding in this tissue have been described earlier (14).

Binding assays. Radioligand binding was performed according to previously described techniques (15, 16). Briefly, membranes were incubated with the radioligands in the absence or presence of various drugs at 25° for 60 min. The incubation was stopped by rapid vacuum filtration through two layers of Whatman GF/C filters using a Brandel cell harvester; filters were rinsed twice with 10 ml of ice-cold binding buffer. Protein was measured by the method of Lowry *et al.* (17).

A slightly different protocol was used for binding to human kidney and myometrial membranes. [³H]YOH was used instead of [³H]RAU, and membranes were incubated for 30 min at 25° with this ligand. In experiments with α-adrenergic agonists, 100 μM GTP was present in all tubes. Incubation of the membranes was stopped by addition of 10 ml of buffer and rapid vacuum filtration through a single layer of Whatman GF/C filters, which were rinsed with an additional 10 ml of buffer.

Data analysis. Concentration-inhibition and saturation curves were analyzed by nonlinear regression analysis with the GraphPAD program (Institute for Scientific Information, Philadelphia, PA). Data from competition experiments were fitted to a sigmoid curve, data from saturation experiments to a rectangular hyperbola. IC_{50} values obtained from the concentration-inhibition curves were converted to K_i values according to the equation

$$K_i = IC_{50}/(L/K_d + 1)$$

where L and K_d are the concentration and the affinity of the radioligand, respectively.

Results

[³H]IDA and [³H]RAU binding in rat renal cortical membranes. In initial experiments conducted with rat renal cortical membranes, we found that [³H]IDA consistently bound to twice as many sites as did [³H]RAU (data not shown). In order to determine whether this might be attributed to additional "nonspecific" and/or "specific" binding, we performed competition experiments with a variety of structurally different drugs.

Total [³H]RAU binding was maximally inhibited 72–87% by each adrenergic compound tested, including the endogenous catecholamines epinephrine and norepinephrine and the selective α_1- and α_2-adrenergic antagonists prazosin and yohimbine, respectively (Fig. 1; Table 1). Thus, approximately 20% of total [³H]RAU binding in rat kidney is not competed for by any of the drugs tested and is, therefore, nonspecific; the definition of specific [³H]RAU binding does not depend on the choice of adrenergic drug. The affinities (K_i values) for inhibition of [³H]RAU binding agree well with previously published results (16, 18). It should be noted that prazosin had a rather high affinity (K_i = 74 nM; Fig. 1E; Table 1), confirming the predominance of α_{2B}-adrenergic receptors in rat kidney (1).

Competition for [³H]IDA binding in rat kidney membranes yielded quite different results. Many compounds including epinephrine and norepinephrine, clonidine, phentolamine, prazosin, and yohimbine competed for only 28–34% of the total [³H]IDA binding (Fig. 1; Table 1). The remaining [³H]IDA binding, however, was not entirely nonspecific, inasmuch as compounds known to interact with α-adrenergic receptors (guanabenz, UK 14,304, tolazoline, SK&F 86,466, SK&F 104,078, and idazoxan) all competed for 66–72% of total [³H]IDA binding with high affinity (i.e., with K_i values of <3 μM; Fig. 1, Table 1). Thus, defining the amount of nonspecific [³H]IDA binding by phentolamine would markedly underestimate specific binding. We, therefore, defined specific binding in further experiments by tolazoline (100 μM). Under these conditions, the catecholamines epinephrine and norepinephrine, as well as several other adrenergic compounds, competed for only half of the specific [³H]IDA binding sites. Differences in lipophilicity cannot explain these data because the lipophilicity of yohimbine and phentolamine is greater than that of idazoxan and UK 14,304 (19). Instead, our data demonstrate that, in rat kidney membranes, [³H]IDA specifically binds to two sites, the α_2-adrenergic receptor and an additional, hitherto unidentified, binding site. Accordingly, the density of specific [³H]IDA binding sites (defined by 100 μM tolazoline) was twice that of α_2-adrenergic receptors as determined by [³H]RAU binding (Table 2).

Although competition binding studies demonstrated that [³H]IDA identified two different binding sites, the saturation isotherm of specific [³H]IDA binding was monophasic (Fig. 2). Fitting the saturation binding data to a two-site model (double rectangular hyperbola) instead of a one-site model did not result in a significantly better fit (data not shown). Moreover, the K_d value for [³H]IDA binding (37 nM) and the K_i values of unlabeled idazoxan inhibiting [³H]RAU (100 nM) and [³H]IDA binding (45 nM) were similar (Tables 1 and 2). Thus, idazoxan seems to have similar affinities for the α_2-adrenergic receptor and the additional binding site.

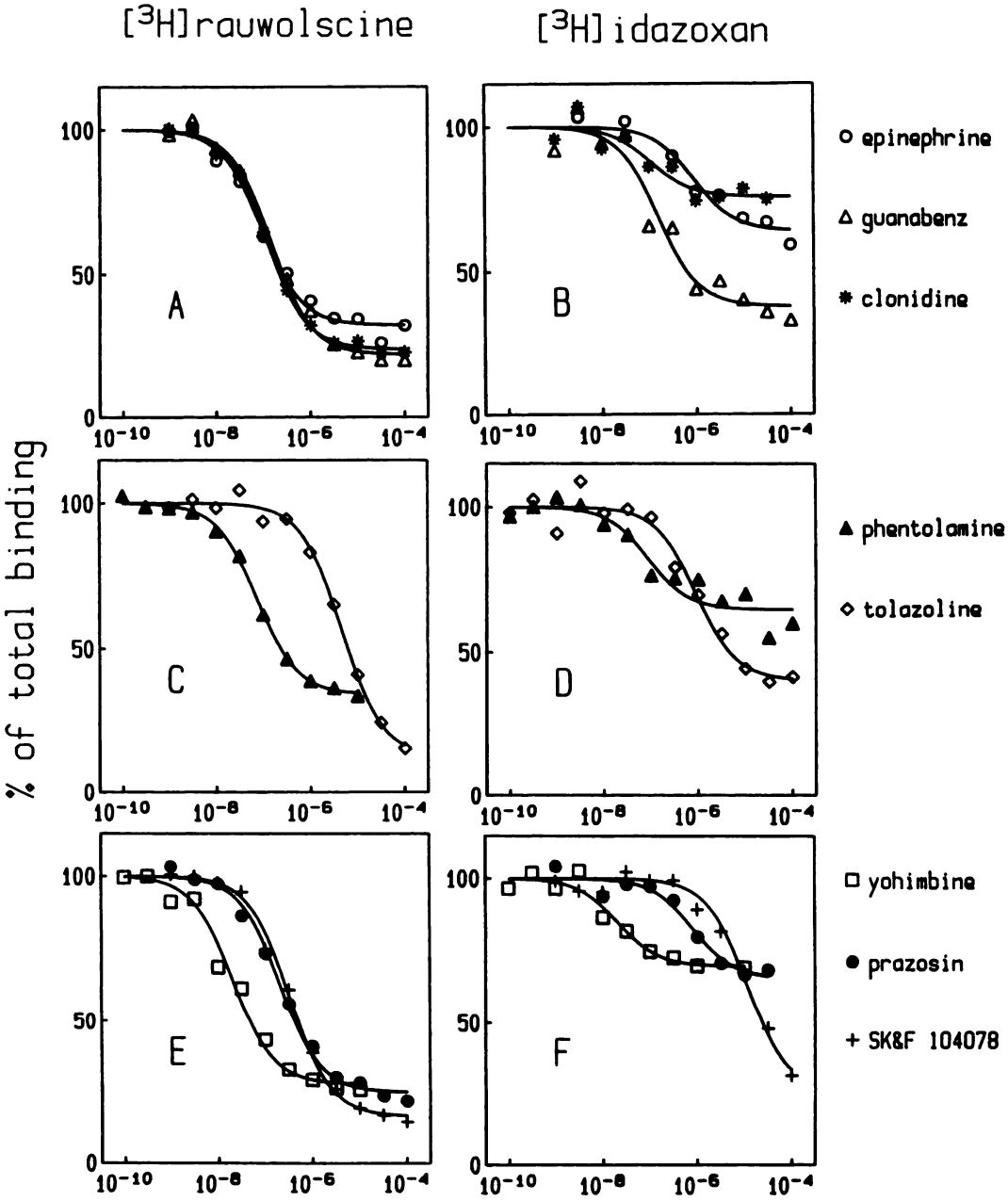

Fig. 1. Inhibition of [^{3}H]RAU and [^{3}H]IDA binding to rat renal cortical membranes. Shown are representative experiments for the inhibition of [^{3}H]RAU (approximately 6 nM; A, C, and E) and [^{3}H]IDA (approximately 30 nM; B, D, and F) binding with each *point* determined in duplicate. A summary of pooled data is given in Table 1.

Most other compounds binding to the additional [^{3}H]IDA site yielded steep and apparently monophasic competitive binding curves, indicating that these drugs also have similar affinities for the α_2-adrenergic receptors and the additional [^{3}H]IDA binding site. For example, the α_2-adrenergic agonist UK 14,304 competed with similar IC_{50} values for [^{3}H]IDA binding (both sites), [^{3}H]IDA binding in the presence of 10 μM phentolamine ("additional" [^{3}H]IDA sites only), and [^{3}H]RAU binding (α_2-adrenergic receptors; Fig. 3). Only the benzazepine SK&F 86,466 had a biphasic competition curve with a high affinity component, similar to its α_2-adrenergic receptor affinity, and a low affinity component. The K_i values for all compounds are summarized in Table 1.

[^{3}H]IDA and [^{3}H]YOH binding in human renal cortical membranes. We next asked whether the additional [^{3}H]IDA binding site is also present in tissues other than rat kidney. We, therefore, compared the binding of the rauwolscine isomer [^{3}H]YOH with that of [^{3}H]IDA in human kidney membranes. Preliminary experiments showed that nonspecific binding varied markedly in this tissue between samples obtained from different patients (20–70%; data not shown). Based on the data obtained in rats and on preliminary experiments, we defined specific [^{3}H]YOH binding as binding in the presence of 10 μM phentolamine and specific [^{3}H]IDA binding as binding in the presence of 100 μM tolazoline. Thus, we determined the amount of specific binding in each experiment; inhibition was expressed as a percentage of specific binding (not as a percentage of total binding).

In human kidney membranes, all compounds tested competed for specific [^{3}H]YOH binding completely, with affinities

TABLE 1

Inhibition of [³H]RAU and [³H]IDA binding to rat kidney

Shown are maximal inhibition (in percentage of total binding) and pK_i values of [³H]RAU and [³H]IDA binding, as calculated from concentration-inhibition curves. All drugs were tested up to at least 100 times the K_i value at α_2-adrenergic receptors. Data are mean ± standard error of 2–5 experiments. Standard error values were not calculated when only two experiments were done. Representative experiments are shown in Fig. 1. The competition curve for SK&F 86,466 was apparently biphasic with a high affinity pK_i of 7.80 ± 0.51 and a low affinity pK_i of 5.69 ± 0.45.

	Maximal inhibition of total binding		pK_i	
	[³H]RAU	[³H]IDA	[³H]RAU	[³H]IDA
	%		M	
α-Adrenergic agonists				
Epinephrine	72 ± 4	30 ± 7	7.26 ± 0.30	6.72 ± 0.05
Norepinephrine	76 ± 1	33 ± 1	7.10 ± 0.25	7.25 ± 0.23
Clonidine	77 ± 2	32 ± 5	7.07 ± 0.25	6.98 ± 0.34
p-NH_2-Clonidine	79	34	7.41	6.61
Oxymetazoline	73 ± 1	39 ± 6	6.71 ± 0.21	6.10 ± 0.33
Moxonidine	73 ± 2	33 ± 0	5.77 ± 0.07	5.39 ± 0.02
UK 14,304	74 ± 3	68 ± 3	7.49 ± 0.18	7.93 ± 0.23
Indanidine	82 ± 2	71 ± 1	5.34 ± 0.09	5.38 ± 0.10
Cirazoline	75	57	6.27	6.96
Guanabenz	75 ± 3	66 ± 4	7.17 ± 0.02	7.06 ± 0.07
α-Adrenergic antagonists				
Prazosin	77 ± 2	33 ± 0	7.13 ± 0.03	5.53 ± 0.34
Yohimbine	75 ± 3	28 ± 2	8.16 ± 0.02	7.55 ± 0.22
Phentolamine	78 ± 6	30 ± 5	7.04 ± 0.48	6.51 ± 0.38
Tolazoline	80 ± 5	68 ± 4	5.92 ± 0.11	6.23 ± 0.03
Idazoxan	79 ± 3	72 ± 2	7.00 ± 0.35	7.35 ± 0.34
SK&F 104,078	87 ± 0	71 ± 4	6.82 ± 0.10	5.39 ± 0.06
SK&F 86,466	72	59	7.54	6.46
Nonadrenergic compounds				
Dopamine	75	*	4.66	<4.5
Serotonin	74 ± 3	*	3.68 ± 0.32	<4
Histamine	80 ± 3	78 ± 4	4.23 ± 0.35	4.41 ± 0.42
Cimetidine	73	*	4.53	<4
Amiloride	75 ± 4	51 ± 6	4.91 ± 0.34	5.11 ± 0.12

* Approximately 30% inhibition at concentrations comparable to full competition at α_2-adrenergic receptors; higher concentrations competed somewhat more, but the extent of maximal competition could not be determined because of low affinity.

(K_i values) typical for the interaction with α_2-adrenergic receptors (Table 3). Confirming earlier data (16), the affinity of prazosin for the human renal α_2-adrenergic receptor was 1.2 μM, indicating the predominance of the α_{2A}-subtype in the human kidney.

As was found in studies with rat kidney membranes, saturation binding isotherms of [³H]IDA to human kidney membranes were monophasic (data not shown). The density of specific [³H]IDA binding sites was 4 times that of α_2-adrenergic receptors, determined by [³H]YOH binding (Table 2). The catecholamines epinephrine and norepinephrine, B-HT 920, clonidine, moxonidine, phentolamine, prazosin, and yohimbine all competed for less than 20% of specific [³H]IDA binding, in concentrations up to 100 times their K_i value, at α_2-adrenergic receptors (Table 3). UK 14,304, oxymetazoline, tolazoline, idazoxan, SK&F 86,466, and SK&F 104,078, however, competed fully for specific [³H]IDA binding (Table 3), demonstrating that the additional [³H]IDA binding site also exists in human kidney.

The affinity of idazoxan for binding in human kidney membranes was higher than in rat kidney (Table 2), but similar affinities were found for [³H]IDA binding (4 nM) and for unlabeled idazoxan competing for [³H]YOH (18 nM) and [³H]IDA binding (11 nM). Most drugs that recognized the additional [³H]IDA site had similar affinities for inhibition of [³H]YOH and [³H]IDA binding (Table 2); only the benzazepines SK&F 86,466 and SK&F 104,078 had lower affinities for the [³H]IDA sites than for the α_2-adrenergic receptors.

In contrast to the rat kidney membranes, high concentrations of clonidine and phentolamine (>1000 times the K_i value at the α_2-adrenergic receptor) could compete for specific [³H]IDA binding completely in the human kidney membranes; the respective K_i values were 29 and 17 μM. High concentrations of norepinephrine (>200 times the K_i value at the α_2-adrenergic receptor) maximally inhibited 55% of specific [³H]IDA binding.

[³H]IDA binding in other tissues with adrenergic receptors. We then asked whether the additional [³H]IDA binding site is also found in other tissues. In human platelets, myometrium, and HEL cells the density of specific [³H]IDA binding sites also exceeded that of α_2-adrenergic receptors detectable using [³H]RAU (Table 2). In contrast, three cell lines that lack α_2-adrenergic receptors (MDCK, BC3H1, Jurkat cells) also did not have saturable high affinity [³H]IDA binding sites (Table 2). In some experiments tolazoline-displaceable [³H]IDA binding was observed in these cell lines, but this increased linearly with ligand concentrations (up to 150 nM) and was not further investigated. Thus, the additional [³H]IDA binding site was observed in four tissues containing α_2-adrenergic receptors and not in three cell lines lacking α_2-adrenergic receptors. However, the ratio between α_2-adrenergic receptors and the specific [³H]IDA binding was inconsistent, varying from 1:2 to 1:7 between tissues (Table 2).

Effects of GTP and Na^+ on [³H]IDA binding. Because agonist binding to α_2-adrenergic receptors in rat renal cortex and other tissues is sensitive to GTP and Na^+ (20, 21), we tested whether binding of the α_2-adrenergic agonist UK 14,304

TABLE 2

Comparison of density and affinity of [³H]IDA and [³H]RAU binding sites

Data are means ± standard error of 2–4 experiments. [³H]YOH was used in experiments with human kidney and human myometrium, [³H]RAU in all other tissues. Specific [³H]RAU and [³H]YOH binding was defined by 10 μM phentolamine, and specific [³H]IDA binding by 100 μM tolazoline.

Tissue	B_{max}		K_D	
	[³H]RAU/[³H]YOH	[³H]IDA	[³H]RAU/[³H]YOH	[³H]IDA
	fmol/mg of protein		nM	
Rat kidney	195 ± 34	385 ± 60	4.70 ± 1.36	37.0 ± 8.2
Human kidney	93 ± 23	413 ± 56	2.16 ± 0.41	3.5 ± 0.6
Human platelets	212 ± 78	358 ± 102	1.38 ± 0.41	10.8 ± 1.8
Human myometrium	47 ± 13	127 ± 42	1.67 ± 0.10	10.0 ± 0.3
HEL cells	52 ± 8	355 ± 76	1.44 ± 0.04	22.1 ± 8.0
MDCK cells	—*	—	—	—
BC3H1 cells	—	—	—	—
Jurkat cells	—	—	—	—

* —, No saturable high affinity binding of either ligand was detected in MDCK, BC3H1, and Jurkat cells.

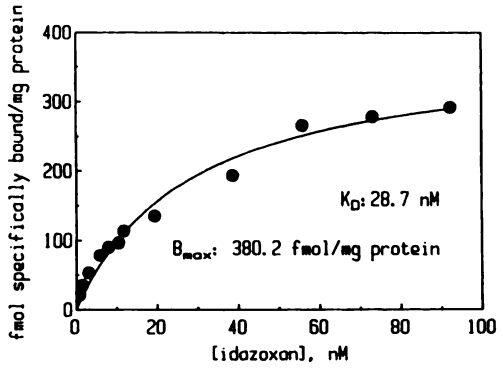

Fig. 2. Saturation isotherm of [^{3}H]IDA binding to rat renal cortical membranes. Shown is a representative experiment with each *point* determined in duplicate. Specific [^{3}H]IDA binding was defined by 100 μM tolazoline. The mean and standard error of the results of the saturation experiments are given in Table 2.

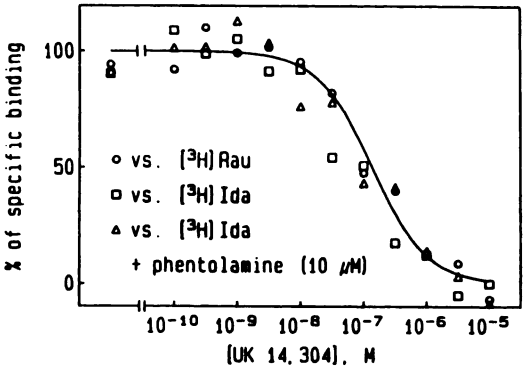

Fig. 3. Competition of UK 14,304 for specific binding of [^{3}H]RAU, [^{3}H]IDA, and [^{3}H]IDA in rat renal cortical membranes in the presence of phentolamine (10 μM, to prevent [^{3}H]IDA binding to α-adrenergic receptors). Specific binding was defined by 10 μM phentolamine for [^{3}H]RAU and 100 μM tolazoline for [^{3}H]IDA binding. The *line* shows the calculated competition curve for [^{3}H]RAU binding. Shown is a representative experiment with each *point* determined in duplicate. Similar results were obtained in two other experiments. Note that the concentration of [^{3}H]RAU in this experiment was 5.5 nM (approximately twice the K_d) and that of [^{3}H]IDA was 28 nM (approximately equal to the K_d).

to the additional [^{3}H]IDA site is also sensitive to these agents. We competed for [^{3}H]IDA (in the presence of 10 μM phentolamine) and [^{3}H]RAU binding with increasing concentrations of UK 14,304 in the absence and presence of Gpp(NH)p (10 μM) and Na^+ (140 mM). Although this combination markedly reduced the affinity of UK 14,304 for the α_2-adrenergic receptor, it did not affect the affinity for the additional [^{3}H]IDA binding site (Fig. 4). Thus, either agonist binding to the additional [^{3}H]IDA binding site is not sensitive to Na^+ and GTP, or UK 14,304 is not an agonist at this site.

Discussion

Idazoxan is an α-adrenergic receptor antagonist that has a higher selectivity for α_2- versus α_1-adrenergic receptors than does either yohimbine or rauwolscine (6, 7). Moreover, in concentrations up to 10 μM it does not antagonize effects mediated via β_1- and β_2-adrenergic, dopamine, neuronal serotonin, nicotine, presynaptic opioid, or histamine H_2 receptors. The pD_2 values at histamine H_1 and muscarinic receptors are very low (4.81 and 4.84, respectively) compared with those at α_2-adrenergic receptors (7.93–8.56; Ref. 6). Thus, idazoxan is

TABLE 3

Inhibition of [^{3}H]IDA and [^{3}H]YOH binding to human kidney

Shown are maximal inhibition (in percentage of specific [^{3}H]IDA binding (as defined by 100 μM tolazoline) and pK_i values for inhibition of [^{3}H]IDA and [^{3}H]YOH binding, as calculated from concentration-inhibition curves. All drugs inhibited specific [^{3}H]YOH binding completely and were tested up to at least 100 times the K_i value at α_2-adrenergic receptors. Data are mean ± standard error of 2–4 experiments. Standard error values were not calculated when only two experiments were done.

	Maximal inhibition of specific binding, [^{3}H]IDA	pK_i	
		[^{3}H]IDA	[^{3}H]YOH
	%	M	
α-Adrenergic agonists			
Epinephrine	14 ± 8	7.27 ± 0.34	6.68 ± 0.27
Norepinephrine	<10[a]	—[b]	6.58 ± 0.32
B-HT 920	<10	—	6.55 ± 0.08
Clonidine	<10[a]	—	7.67 ± 0.16
Moxonidine	19 ± 7	5.80 ± 0.28	5.65 ± 0.16
UK 14,304	96 ± 7	6.17 ± 0.33	6.92 ± 0.12
Oxymetazoline	102	6.34	6.96
α-Adrenergic Antagonists			
Prazosin	<10	—	5.92 ± 0.03
Yohimbine	<10	—	8.40 ± 0.10
Phentolamine	<10[a]	—	8.21 ± 0.17
Tolazoline	105 ± 8	6.22 ± 0.34	5.80 ± 0.07
Idazoxan	108	7.96	7.74
SK&F 104,078	99 ± 4	5.20 ± 0.32	6.94 ± 0.26
SK&F 86,466	100	5.31	7.55

[a] Less than 10% inhibition of [^{3}H]IDA binding in concentrations up to 100 times the respective K_i values at α_2-adrenergic receptors; higher concentrations partially (norepinephrine) or fully (clonidine and phentolamine) competed for [^{3}H]IDA binding.
[b] —, Could not be determined reliably.

considered to be one of the most selective and specific α_2-adrenergic drugs known. Therefore, we were surprised to find that, as a radioligand, [^{3}H]IDA consistently labeled more sites than did [^{3}H]RAU or [^{3}H]YOH in membranes from rat and human kidney, human platelets, human myometrium, and HEL cells (Table 3). The additional [^{3}H]IDA binding sites are not nonspecific because they are saturable and competed by structurally distinct drugs with high affinity.

The catecholamines epinephrine and norepinephrine only competed for part of the specific [^{3}H]IDA binding (Fig. 1B, Tables 1 and 3). The fraction of catecholamine-displaceable [^{3}H]IDA binding was similar to the ratio of the B_{max} of [^{3}H]RAU (or [^{3}H]YOH) to that of [^{3}H]IDA binding. Thus, the additional [^{3}H]IDA binding sites do not appear to be catecholamine recognition sites and, thus, can be classified as nonadrenergic. They also do not appear to interact with the α-adrenergic drugs clonidine, p-NH_2-clonidine, moxonidine, B-HT 920, prazosin, yohimbine, or phentolamine (Tables 1 and 3), or with serotonin or dopamine (in concentrations up to 0.1 mM). Histamine inhibited [^{3}H]IDA binding with an affinity that is not greater than that observed at α_2-adrenergic receptors. Thus, the additional sites that bind [^{3}H]IDA appear not likely to be receptor binding sites for any of those neurotransmitters.

Although the additional [^{3}H]IDA binding site is not recognized by catecholamines, it is competed for by many α-adrenergic drugs including UK 14,304, indanidine, SK&F 86,466, SK&F 104,078, tolazoline, guanabenz, idazoxan, and (in human kidney membranes) oxymetazoline (Tables 1 and 2). Cirazoline competed for specific [^{3}H]IDA binding in rat kidney to a significantly greater extent than did the catecholamines, but complete competition was not achieved (Table 1); in a single experiment in human kidneys, cirazoline completely inhibited specific [^{3}H]IDA binding (data not shown).

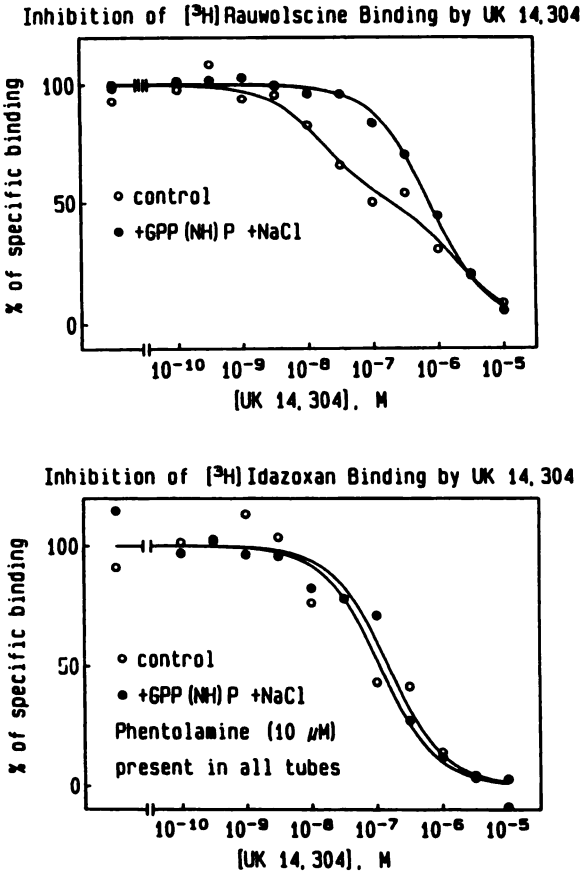

Fig. 4. Effect of Gpp(NH)p and Na^+ on binding of UK 14,304 to α_2-adrenergic receptors ([³H]RAU binding; *upper*) and the additional [³H]IDA binding sites (in the presence of 10 μM phentolamine; *lower*) in rat renal cortical membranes. Experiments were performed in the absence and presence of Gpp(NH)p (10 μM) and Na^+ (140 mM). The concentrations of [³H]IDA and [³H]RAU were approximately 30 and 6 nM, respectively. For these experiments membranes had been washed three times for 20 min at 40,000 × *g* to remove endogenous GTP. $MgCl_2$ (5 mM) was added to all tubes. Specific binding was defined by 10 μM phentolamine for [³H] RAU and 100 μM tolazoline for [³H]IDA binding. Shown is a representative experiment with each *point* determined in duplicate. Similar results were obtained in two other experiments.

Because many α-adrenergic drugs had high affinity for the additional [³H]IDA binding site, we asked whether this site might be an allosteric site on the α_2-adrenergic receptor. α_2-Adrenergic receptors appear to have at least one allosteric binding site that is recognized by the diuretic amiloride (21, 22). Amiloride, however, did not compete for the additional [³H]IDA binding sites in rat kidney (Table 1).

The additional [³H]IDA site also exists in four tissues containing α_2-adrenergic receptors, but not in three cell lines devoid of α_2-adrenergic receptors (Table 2). The ratio of α_2-adrenergic receptors/[³H]IDA binding sites was not constant and varied from 1:2 to 1:7. Experiments designed to test whether the additional [³H]IDA site could be detected on purified α_2-adrenergic receptors (kindly performed by Drs. J. M. Nunnari and L. E. Limbird, Vanderbilt University, Nashville, TN) were unsuccessful because [³H]IDA did not detect either α_2-adrenergic or additional binding, possibly because of the low affinity. Thus, we could not determine whether the additional [³H]IDA binding site is part of or closely affiliated with the α_2-adrenergic receptor molecule, although the close correspondences between tissues makes this an intriguing hypothesis.

Recently, an extract has been isolated from brain that displaces [³H]*p*-NH_2-clonidine binding and has therefore been termed CDS (23). CDS binding sites have been identified in rat brain (23, 24) and kidney (25). Many α-adrenergic drugs have high affinity for the CDS site, and their potency for lowering blood pressure correlates better with their affinity for CDS sites than with that for α-adrenergic receptors (26). However, a number of drugs distinguish the additional [³H]IDA site described here from the CDS site. 1) The CDS site is defined via [³H]*p*-NH_2-clonidine binding (23, 24) but neither this drug, nor the parent compound clonidine, recognized the additional [³H]IDA site (Fig. 1B; Tables 1 and 3). 2) Histamine and the histamine H_2-receptor antagonist cimetidine have higher affinity for CDS sites than for α_2-adrenergic receptors (26), but the affinity of histamine and cimetidine for the additional [³H]IDA site is similar to or lower than that for α_2-adrenergic receptors (Table 1). 3) Yohimbine competes for binding to CDS sites (26) but not to the additional [³H]IDA site. 4) Guanabenz does not bind to CDS sites but had high affinity for the additional [³H] IDA site (26). 5) Generally, all imidazolines bind to CDS sites (26), whereas the additional [³H]IDA site is recognized by some imidazolines (UK 14,304, indanidine, cirazoline, oxymetazoline, tolazoline, and idazoxan) but not by others (clonidine, *p*-NH_2-clonidine, moxonidine, and phentolamine). On the other hand, nonimidazolines appear not to bind to CDS sites, whereas the benzazepines SK&F 86,466 and SK&F 104,078 recognize the additional [³H]IDA sites in rats and humans.

In this context, it should be noted that evidence has recently been presented for an additional binding site of [³H]IDA in rabbit kidney and brain (27–29). This site is also not detected by catecholamines, prazosin, yohimbine, and rauwolscine but is readily detected by several imidazolines including tolazoline, tramazoline, clonidine, and phentolamine. The authors of these communications, therefore, concluded that in the rabbit [³H] IDA identifies an imidazoline recognition site that may be closely related to the CDS binding site. The additional [³H] IDA site that we have identified in rat and human kidney, however, is not readily recognized by a number of imidazolines including phentolamine and clonidine and, thus, appears to be pharmacologically different from the site in rabbit kidney and brain.

In summary, in several tissues [³H]IDA binds to two sites; the α_2-adrenergic receptor and a previously unreported additional site. Whether this additional binding site has physiological relevance remains to be determined. However, drugs that interact at the additional [³H]IDA binding site have physiological effects that do not appear to be mediated by α_2-adrenergic receptors. 1) In isolated tubule cells from rat kidney, guanabenz decreases oxygen consumption. This is not mimicked by clonidine and is not blocked by yohimbine.[1] 2) SK&F 104.078 (in concentrations up to 10 μM) is inactive against the inhibitory presynaptic effect of the α_2-adrenergic agonist B-HT 933 in the guinea pig atrium but antagonizes that of oxymetazoline with a K_b of approximately 1 μM.[2] This result is in good agreement with the K_i of SK&F 104,078 at [³H]IDA binding sites (Tables 1 and 3). 3) The first thorough report on the pharmacology of idazoxan demonstrated that the inhibitory effect of guanabenz

[1] M. Zeidel, personal communication.
[2] A. Sulpizio and J. P. Hieble, personal communication.

on the stimulation-induced contraction of rat anococcygeus muscle was reversed by idazoxan but not by phentolamine, piperoxan, or prazosin (6).

Regardless of its physiological role, which will require further studies, the identification of an additional class of sites for [^{3}H] IDA is important for further studies of α_2-adrenergic receptors. Because the additional [^{3}H]IDA sites are recognized with high affinity by many of the probes used to subclassify α_2-adrenergic receptors, findings in this report emphasize that great care must be taken when oxymetazoline, SK&F 104.078, and [^{3}H] IDA itself are used for α_2-adrenergic receptor subclassification. Moreover, the current results also suggest that use of [^{3}H]IDA to define CDS sites or imidazoline sites also may be fraught with potential difficulty.

References

1. Bylund, D. B. Heterogeneity of *alpha*-2 adrenergic receptors. *Pharmacol. Biochem. Behav.* **22**:835–843 (1985).
2. Bylund, D. B., C. Ray-Prenger, and T. J. Murphy. *Alpha*-2A- and *alpha*-2B-adrenergic receptor subtypes: antagonist binding in tissues and cell lines containing only one subtype. *J. Pharmacol. Exp. Ther.* **245**:600–607 (1988).
3. Kobilka, B. K., H. Matsui, T. S. Kobilka, T. L. Yang-Feng, U. Francke, M. G. Caron, and R. J. Lefkowitz. Cloning, sequencing, and expression of the gene coding for the human platelet α_2-adrenergic receptor. *Science* (Wash. D. C.) **238**:650–656 (1987).
4. Regan, J. W., T. S. Kobilka, T. L. Yang-Feng, M. G. Caron, R. J. Lefkowitz, and B. K. Kobilka. Cloning and expression of a human kidney cDNA for an α_2-adrenergic receptor subtype. *Proc. Natl. Acad. Sci. USA* **85**:6301–6305 (1988).
5. Ruffolo, Jr., R. R., A. C. Sulpizio, A. J. Nichols, R. M. DeMarinis, and J. P. Hieble. Pharmacological differentiation between pre- and postjunctional α_2-adrenoceptors by SK&F 107,078. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **336**:415–418 (1987).
6. Doxey, J. C., A. G. Roach, and C. F. C. Smith. Studies on RX 781094: a selective, potent and specific antagonist of α_2-adrenoceptors. *Br. J. Pharmacol.* **78**:489–505 (1983).
7. Ruffolo, Jr., R. R., R. M. DeMarinis, M. Wise, and J. P. Hieble. Structure-activity relationships for *alpha*-2-adrenergic receptor agonists and antagonists, in *The alpha-2-Adrenergic Receptors* (L. E. Limbird ed.), Humana Press, Clifton, NJ, 115–186 (1988).
8. Boyajian, C. L., and F. M. Leslie. Pharmacological evidence for *alpha*-2-adrenoceptor heterogeneity: differential binding properties of [^{3}H]rauwolscine and [^{3}H]idazoxan in rat brain. *J. Pharmacol. Exp. Ther.* **241**:1092–1098 (1987).
9. Boyajian, C. L., S. E. Loughlin, and F. M. Leslie. Anatomical evidence for *alpha*-2-adrenoceptor heterogeneity: differential autoradiographic distributions of [^{3}H]rauwolscine and [^{3}H]idazoxan in rat brain. *J. Pharmacol. Exp. Ther.* **241**:1079–1091 (1987).
10. McKernan, R. M., M. J. Howard, H. J. Motulsky, and P. A. Insel. Compartmentation of α_2-adrenergic receptors in human erythroleukemia (HEL) cells. *Mol. Pharmacol.* **32**:258–265 (1987).
11. Slivka, S. E., and P. A. Insel. *Alpha*$_1$-adrenergic receptor-mediated phosphoinositide hydrolysis and prostaglandin E_2 formation in Madin-Darby canine kidney cells. *J. Biol. Chem.* **262**:4200–4207 (1987).
12. Hughes, R. J., and P. A. Insel. Agonist-mediated regulation of *alpha*$_1$- and *beta*$_2$-adrenergic receptor metabolism in a muscle cell line, BC3H1. *Mol. Pharmacol.* **29**:521–530 (1986).
13. Michel, M. C., X. L. Wang, E. Schlicker, M. Göthert, J. J. Beckeringh, and O.-E. Brodde. Increased β_2-adrenoceptor density in heart, lung and kidney of spontaneously hypertensive rats. *J. Auton. Pharmacol.* **7**:41–51 (1987).
14. Brodde, O.-E., U. Seher, M. Nohlen, M. W. Fischer, and M. C. Michel. Correlation between myometrial and platelet α_2-adrenoceptor density. *Eur. J. Pharmacol.* **150**:403–404 (1988).
15. Howard, M. J., M. D. Mullen, and P. A. Insel. Amiloride interacts with renal α- and β-adrenergic receptors. *Am. J. Physiol.* **253**:F21–F25 (1987).
16. Neylon, C. B., and R. J. Summers. [^{3}H]Rauwolscine binding to α_2-adrenoceptors in the mammalian kidney: apparent receptor heterogeneity between species. *Br. J. Pharmacol.* **85**:349–359 (1985).
17. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
18. Cheung, Y.-D., D. B. Barnett, and S. R. Nahorski. Heterogeneous properties of *alpha*$_2$-adrenoceptors in particulate and soluble preparations of human platelet and rat and rabbit kidney. *Biochem. Pharmacol.* **35**:3767–3755 (1986).
19. McKernan, R. M., W. R. Strickland, and P. A. Insel. Selective blockade and recovery of cell surface α_2-adrenergic receptors in human erythroleukemia cells (HEL) cells: studies with the irreversible antagonist henextramine. *Mol. Pharmacol.* **33**:51–57 (1988).
20. Snavely, M. D., and P. A. Insel. Characterization of *alpha*-adrenergic receptor subtypes in rat renal cortex: differential regulation of *alpha*$_1$- and *alpha*$_2$-receptors by guanyl nucleotides and Na^+. *Mol. Pharmacol.* **22**:532–546 (1982).
21. Nunnari, J. M., M. G. Repaske, S. Brandon, E. J. Cragoe, Jr., and L. E. Limbird. Regulation of porcine brain α_2-adrenergic receptors by Na^+, H^+ and inhibitors of Na^+/H^+ exchange. *J. Biol. Chem.* **262**:12387–12392 (1987).
22. Howard, M. J., R. J. Hughes, H. J. Motulsky, M. D. Mullen, and P. A. Insel. Interactions of amiloride with α- and β-adrenergic receptors: amiloride reveals an allosteric site on α_2-adrenergic receptors. *Mol. Pharmacol.* **32**:53–58 (1987).
23. Ernsberger, P., M. P. Meeley, and D. J. Reis. An endogenous clonidine-like substance binds preferentially to imidazole binding sites in the ventrolateral medulla labeled by ^{3}H-*para*-aminoclonidine. *J. Hypertens.* **4** (Suppl 5) S109–S111 (1986).
24. Ernsberger, P., M. P. Meeley, and D. J. Reis. An endogenous substance with clonidine-like properties: selective binding to imidazole sites in the ventrolateral medulla. *Brain Res.* **441**:309–318 (1988).
25. Ernsberger, P., G. Feinland, M. P. Meeley, and D. J. Reis. In rat renal cortex, clonidine binds to imidazole sites as well as α_2-adrenergic receptors. *Am. J. Hypertens.* **1**:30A–31A (1988).
26. Ernsberger, P., R. Giuliano, R. N. Willette, A. R. Granata, and R. J. Reis. Hypotensive action of clonidine analogs correlates with binding affinity at imidazole and not α_2-adrenergic receptors in the rostral ventrolateral medulla. *J. Hypertens.*, **6** (Suppl. 4): S554–S557 (1988).
27. Coupry, I., R. A. Podevin, J.-P. Dausse, and A. Parini. Evidence for imidazoline binding sites in basolateral membranes from rabbit kidney. *Biochem. Biophys. Res. Commun.* **147**:1055–1060 (1987).
28. Hamilton, C. A., J. L. Reid, and M. A. Yakubu. [^{3}H]Yohimbine and [^{3}H] idazoxan bind to different sites on rabbit forebrain and kidney membranes. *Eur. J. Pharmacol.* **146**:345–348 (1988).
29. Lachaud, V., I. Coupry, R. A. Podevin, J. P. Dausse, E. Koenig, and A. Parini. Interaction of central *alpha*-2 adrenergic agonists with imidazoline preferring receptors. *Am. J. Hypertens.* **1**:39A (1988).

Send reprint requests to: Dr. Martin C. Michel, Dept. of Pharmacology M-036, University of California San Diego, La Jolla, CA 92093.