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NON-ADRENERGIC BINDING SITES FOR THE "α_2-ANTAGONIST" [^{3}H]IDAZOXAN IN THE RABBIT URETHRAL SMOOTH MUSCLE

PHARMACOLOGICAL AND BIOCHEMICAL CHARACTERIZATION

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Abstract—In the present study, pharmacological and biochemical binding characteristics of [^{3}H]idazoxan, an originally thought α_2-adrenoceptor antagonist, have been determined in smooth muscle of rabbit urethra. It is shown that [^{3}H]idazoxan labels with high affinity non-adrenergic binding sites. Specific binding of [^{3}H]idazoxan is inhibited by compounds possessing an imidazoline or a guanidinium moiety whereas phenylethanolamines and classical α_2-antagonists are ineffective competitors which suggests an imidazoline-preferring binding site. However, imidazolidines such as clonidine and *p*-aminoclonidine are poorly effective, which differs considerably from pharmacological characteristics of imidazoline binding sites previously reported in the central nervous system. In addition, it is shown that K^+ and Mn^{2+} inhibit [^{3}H]idazoxan binding in a competitive and non-competitive manner, respectively. Other cations such as Na^+, Li^+ and Mg^{2+} have no significant effect. It is shown that K^+ accelerates the dissociation of [^{3}H]idazoxan binding while Mn^{2+} does not produce any modification. These results suggest that K^+ may bind to an allosteric site, while Mn^{2+} may bind with a membrane component susceptible to alter [^{3}H]idazoxan binding sites.

The cation regulation of binding has been largely investigated to clarify molecular properties of α_2-adrenoceptors [1]. It has been shown that the sodium ion increased *in vitro* α_2-adrenoceptor densities in rat cerebral [2] and renal cortex [3]. In addition, other reports have shown that Na^+ diminished *in vitro* the affinity of α_2-adrenoceptors for agonists [4–8]. The effect of sodium was specific since it was observed that other monovalent cations (Li^+ and K^+) were much less effective. It has been proposed that Na^+ modulated the binding of agonists to α_2-adrenoceptors by acting on an allosteric site [9]. In contrast, divalent cations such as Mg^{2+} and Mn^{2+} increased apparently binding affinity by decreasing the rate of dissociation and also by increasing the rate of association [8].

A wide spectrum of molecules as derivatives of imidazolines, imidazolidines, guanidines and others, have been largely used to investigate functional and biochemical characteristics of α_2-adrenoceptors [1]. However, it has been reported recently that certain pharmacological effects elicited by some α_2-agonists in the central nervous system might be mediated by non-adrenergic receptors. For instance, an imidazoline receptor was now thought to be responsible for the central hypotensive action of clonidine and of other imidazolines [10]. The new imidazoline compound, idazoxan, was previously described as a preferential peripheral and central α_2-adrenoceptor antagonist [11]. However, it was reported that idazoxan might recognize, in addition to α_2-adrenoceptors, non-adrenergic binding sites in the rabbit renal proximal tubule which could be related to imidazoline-preferring receptors [12].

In contrast, we provided recently further evidence that [^{3}H]idazoxan could bind preferentially only to non-adrenergic sites in rabbit urethral smooth muscle [13]. This finding has been confirmed by others in rabbit forebrain and kidney [14], rabbit adipocytes [15] and pig kidney [16]. In addition, more recent studies conducted with guanidine derivatives reported no relevance between peripheral [^{3}H]-idazoxan binding sites [17, 18] and the previously described central imidazoline receptor [19].

In order to progress further in the determination of the pharmacological characteristics of urethral smooth muscle sites recognized by [^{3}H]idazoxan, we studied the interaction with a series of imidazoline, imidazolidine and non-imidazolidine compounds. Moreover, in the attempt to clarify their molecular properties, we tested their regulation by mono- and divalent cations.

MATERIALS AND METHODS

Materials. [^{3}H]2-(2-(1,4-Benzodioxanyl)-2-imidazolin HCl, [^{3}H]idazoxan or [^{3}H]RX781094 (sp. act 53–60 Ci/mmol) was obtained from Amersham, U.K. The following drugs were supplied by the indicated companies: idazoxan (Reckitt and Colman), rauwolscine (Carl Roth), UK-14,304 (Pfizer), cirazoline (L.E.R.S), WB 4101 (Amersham), phentolamine (Ciba-Geigy), benzamil and *N*-ethylisopropyl amiloride (Merck Sharp

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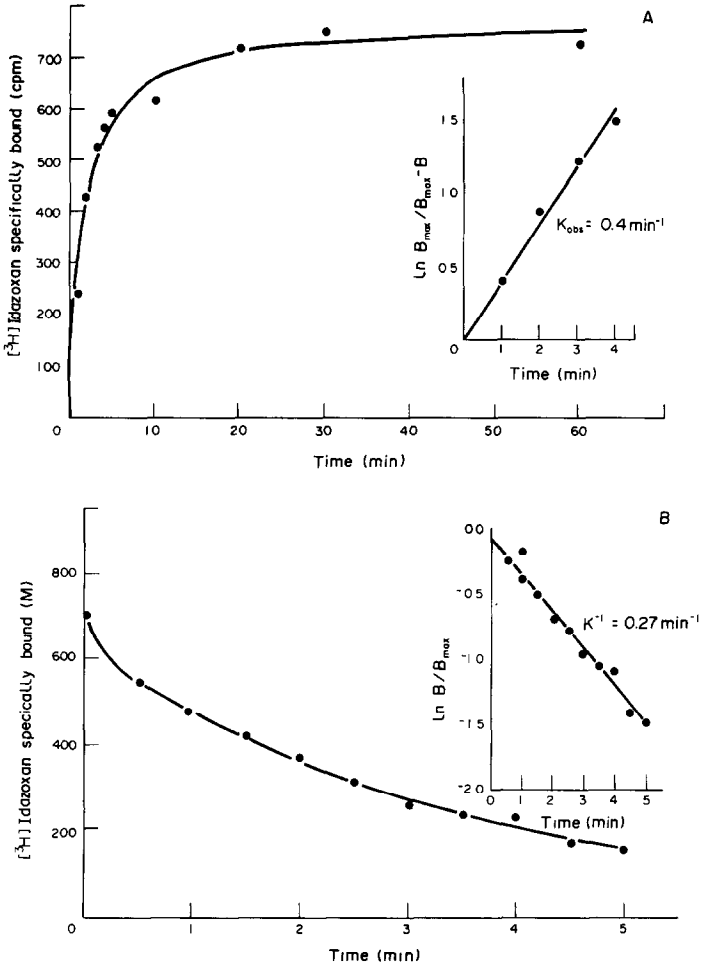

Fig. 1. Kinetic characteristics of the specific binding of [^{3}H]idazoxan to rabbit urethral membranes. (A) Time course for the association reaction. Urethral membranes were incubated at 28° with 2 nM [^{3}H]idazoxan and specific binding was determined in duplicate at different indicated times; inset: pseudo-first order kinetic plot of association curves where B and B_{max} represent the binding at time t and at equilibrium. The slope represent the pseudo-first order association rate constant (k_{obs}). (B) Time course of the dissociation reaction. Urethral membranes were incubated for 30 min at 28° with 2 nM [^{3}H]idazoxan, and a large excess of phentolamine (25 μM) was added at $t = 0$. The specific binding of [^{3}H]idazoxan was determined at various times and plotted as a function of time; inset: first order kinetic plot. Data shown are representative of one experiment performed in triplicate.

and Dohme Research Laboratories), ketanserine, spiperone, butaclamol and *p*-aminoclonidine (Research Biochemicals Inc.), St-587 (Boehringer Ingelheim), St-1059 (C.L. Pharma), Sgd-10175 (Siegfried Pharma), guanabenz (Wyeth-Byla) and guanfacine (Sandoz).

All other compounds were obtained from the Sigma Chemical Co.

Membrane preparation. Male New Zealand rabbits (average weight approximately 3 kg) were killed by an intravenous air injection via an ear vein. The urethra extending from the bladder base through the penis were rapidly removed and opened longitudinally. Mucosal and serosal layers were discarded. For each binding experiment, the urethra from at least two animals was carefully minced and homogenized with a Polytron PT/10 homogenizer for 3 × 10 sec (setting 10) in ice-cold 10 mM Tris–HCl, 1 mM K-EDTA, 0.32 M sucrose, pH 7.4, at 25°.

The homogenate was filtered through a double layer of gauze and centrifuged at 500 *g* for 10 min at 4° in a Sorvall RC5C. The supernatant was then centrifuged at 50,000 *g* for 20 min. The pellet was resuspended in 50 mM Tris–HCl, 50 μM K-EDTA, pH 7.4, and centrifuged as before.

The final pellet was suspended in the same buffer for the binding assay. Protein concentrations was determined by the method of Lowry *et al.* [20] using bovine serum albumin as standard.

Binding assays. Incubations were performed in duplicate at 28° for 30 min in a total volume of 0.3 mL, consisting of [^{3}H]idazoxan and a suspension of urethral membranes (approximately 0.3–0.4 mg

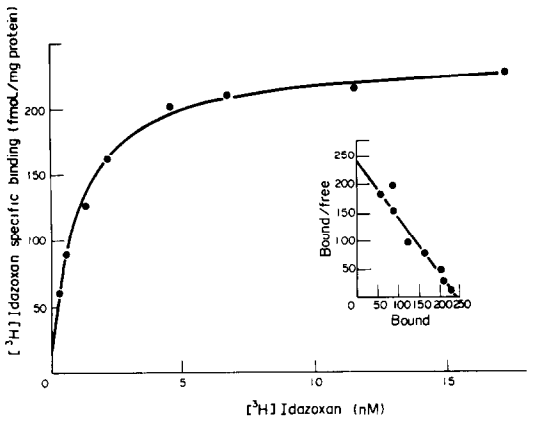

Fig. 2. [^{3}H]Idazoxan specific binding to rabbit urethral membranes. Urethral membranes were incubated with increasing concentrations of [^{3}H]idazoxan. Non-specific binding was determined with 25 μM phentolamine. In inset: Scatchard plot of specific binding isotherm (B_{max} = 239 fmol/mg protein; K_d = 1.2 nM). These results are representative of one experiment performed in duplicate.

protein/mL), either with buffer alone (total binding) or with buffer containing 25 μM phentolamine (non-specific binding). Incubations were terminated by adding 2 mL of ice-cold incubation buffer followed by immediate filtration over glass-fiber filters (Whatman GF/C) presoaked at 4° with 0.1% polyethyleneimine. The filters were then washed with 20 mL of cold incubation buffer and the radioactivity was counted by scintillation spectrophotometry at 60% efficiency.

In the presence of 25 μM phentolamine, the non-specific binding represented 25% of total binding at ligand concentrations around 2 nM. The same amount of non-specific binding was obtained using 25 μM naphazoline.

Data from saturation and competition studies were analysed using a non-linear least square curve fitting procedure [21] adapted for IBM PC computers (Elsevier BIOSOFT, Cambridge, U.K.) [22]. Results are expressed as the means ± SE. Statistical analysis of data was carried out with the non-parametric Krushal–Wallis test (ANOVA).

Differences are reported as significant at P values less than 0.05.

Table 1. Effect of various drugs on [^{3}H]idazoxan binding in rabbit urethral membranes

Competitor	K_i (nM)
Imidazolines	
Idazoxan	1.63 ± 0.16*
Cirazoline	2.90 ± 0.66*
Naphazoline	4.80 ± 0.85*
Tymazoline	15 ± 2
Tolazoline	130.6 ± 58.0*
Phentolamine	455 ± 75*
Oxymetazoline	4431 ± 148*
Imidazolidines	
Tramazoline	34.6 ± 10.9
St-587	74.6 ± 11.8
UK-14,304	297 ± 17*
Sgd-10175	339 ± 65
Clonidine	1265 ± 168*
p-Aminoclonidine	1743 ± 970*
Guanidines	
Guanabenz	4.46 ± 0.95†
Amiloride	30.3 ± 6.4†
N-Ethylisopropylamiloride	38 ± 2†
Benzamil	83 ± 2†
Guanfacine	701 ± 217
Guanethidine	1046 ± 99
Imidazoles	
Histamine	>20,000*
Cimetidine	>20,000
Phenylethanolamines	
(−)Adrenaline	>20,000*
(−)Noradrenaline	>20,000*
(±)α-Methyl noradrenaline	>20,000*
Other adrenergic compounds	
Yohimbine	5818 ± 1648*
Rauwolscine	>20,000*
Prazosine	4086 ± 1729*
WB 4101	1078 ± 91
St-1059	2719 ± 422
Ketanserin	2169 ± 521*
Propranolol	3888 ± 824*
Other non-adrenergic compounds	
Spiperone	661 ± 181
(+) Butaclamol	706 ± 45
Dopamine	>20,000*
Nifedipine	>20,000
Diltiazem	>20,000

The effect of displacing agents were studied using [^{3}H]idazoxan (2 nM). Each drug was tested in duplicate at concentrations varying from 0.1 to 10 mM. Each value is the mean ± SE of three to five experiments.
* From Ref. 13; † From Ref. 17.

RESULTS

General properties of [^{3}H]*idazoxan binding in urethral membranes*

Association of [^{3}H]idazoxan binding (2 nM) to urethral membranes at 28° was rapid, reaching steady state in less than 10 min (Fig. 1A). The equilibrium was maintained up to 60 min, indicating that neither the ligand nor the binding sites were degraded. The mean k_{obs} value was 0.44 ± 0.06 min^{-1} (N = 4). [^{3}H]Idazoxan binding was reversed when 25 μM phentolamine was added after steady state was reached (Fig. 1B). The plot of $\ln[B/B_{eq}]$ versus time was linear. The rate constant of dissociation (k_{-1}) was 0.257 ± 0.02 min^{-1} (N = 5). The second order rate constant for association (k_{+1}) of [^{3}H]idazoxan binding was determined from the equation $k_{+1} = [k_{obs} - (k_{-1})]/[\text{radioligand}]$ to be 0.073 min^{-1} nM^{-1}. The equilibrium dissociation constant (K_d) determined from the ratio k_{-1}/k_{+1} was 3.5 nM. Specific binding of [^{3}H]idazoxan (0.3–17 nM) was a saturable process with saturation occurring at about 8 nM (Fig. 2). The Scatchard plot (Fig. 2, inset) for binding was monophasic, suggesting only one component of binding, for which the approximate K_d value was 1.2 nM. In all experiments, we observed only one class of high affinity sites (K_d = 2.3 ± 0.2 nM) with a capacity of 232 ± 19 fmol/mg protein (N = 10). No

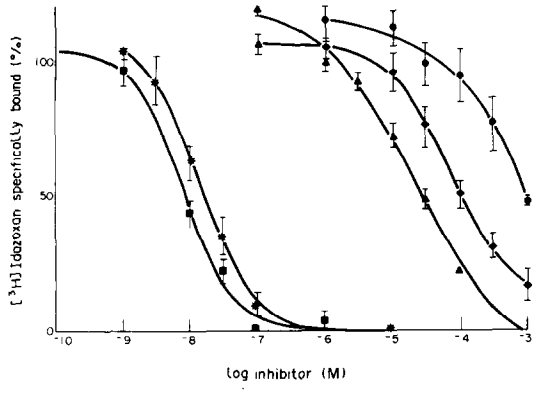

Fig. 3. Inhibition of [^{3}H]idazoxan specific binding to rabbit urethral membranes by idazoxan (■), naphazoline (*), yohimbine (▲), rauwolscine (◆) and adrenaline (●). Urethral membranes were incubated at 28° for 30 min with 2.5 nM [^{3}H]idazoxan. Each point is the mean ± SE of three to five experiments performed in duplicate.

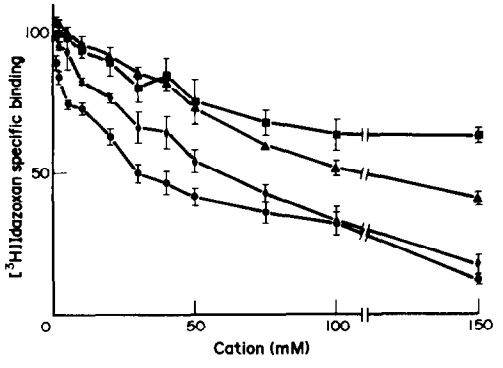

Fig. 4. Inhibition of [^{3}H]idazoxan specific binding by monovalent and divalent cations. The effect of increasing concentrations of Na^+ (■), Li^+ (▲), K^+ (◆) and Mn^{2+} (●) was determined at 2.4 nM [^{3}H]ligand for 30 min at 28°. Each point is the mean ± SE of three different experiments.

evidence of cooperativity was found for these sites since the Hill coefficient was not significantly different from unity (1.01 ± 0.03). Saturation experiments performed in the presence or in the absence of 100 nM rauwolscine or yohimbine to saturate α_2-adrenoceptors did not show any change in [^{3}H]idazoxan binding affinity and capacity (not shown). This result suggest that [^{3}H]idazoxan binds only to non-adrenergic sites.

The specificity of [^{3}H]idazoxan binding was studied in urethral membranes with a large series of compounds selective or not for adrenergic receptors (Table 1).

Figure 3 shows the inhibition of [^{3}H]idazoxan binding by naphazoline, yohimbine, rauwolscine and (−)adrenaline for the [^{3}H]idazoxan binding sites.

Interestingly, the binding of [^{3}H]idazoxan was inhibited by imidazoline analogs independently of their specificity for either α_1- or α_2-adrenoceptors. Indeed, cirazoline, selective for α_1-adrenoceptors, as well as naphazoline and idazoxan, selective for α_2-adrenoceptors, showed high affinity for [^{3}H]idazoxan binding sites. In addition imidazolidine compounds such as clonidine and UK-14,304 affected [^{3}H]idazoxan binding with lower potencies than imidazolines (idazoxan, cirazoline, naphazoline).

In contrast, non-imidazoline adrenergic compounds such as prazosin (α_1) yohimbine and rauwolscine (α_2) and catecholamines did not affect [^{3}H]idazoxan binding.

The lack of any effect of histamine, dopamine and other compounds rules out the possibility that the [^{3}H]idazoxan binding is not a classical known receptor.

Because the imidazoline ring may represent a constrained guanidium group, we tested the effect of guanidine derivatives such as guanabenz, guanfacine and amiloride analogs. All these compounds also inhibited [^{3}H]idazoxan binding with different affinities.

Effect of Gpp(NH)p

GTP and its non-hydrolysable analog Gpp(NH)p are known to diminish the affinity of agonists for the α_2 adrenoceptors.

Addition of increasing concentrations (10^{-9}–10^{-3} M) of Gpp(NH)p did not modify [^{3}H]idazoxan specific binding.

Displacement studies were repeated in the presence of 0.1 mM Gpp(NH)p for some drugs. Gpp(NH)p failed to alter the inhibition curves for guanabenz, Sgd 10175, tymazoline, St 1059, tramazoline, oxymetazoline or phentolamine, suggesting that these drugs did not act as agonists at [^{3}H]idazoxan binding sites, and that these sites are not associated with a GTP binding protein.

Regulation by mono- and divalent cations

In order to investigate the effect of mono- and divalent cations on [^{3}H]idazoxan binding to urethral membranes, we performed binding studies in the absence and in the presence of increasing ion concentrations.

As shown in Fig. 4, [^{3}H]idazoxan binding was inhibited in a dose-dependent manner by K^+ with a maximal inhibition (80%) occurring at 150 mM. The half-maximal effective K^+ concentration was 60 mM.

In contrast, the inhibition of [^{3}H]idazoxan binding was less marked with Li^+ and Na^+ ions (50 and 25%, respectively, at 150 mM concentration).

K-nitrate and K-sulfate decreased binding activity similarly to K-chloride (not shown) which suggests that chloride ions are not responsible for the effect of KCl.

The effect of divalent cations was also investigated with increasing concentrations of $MgCl_2$, $CaCl_2$ or $MnCl_2$ (Fig. 4). Only $MnCl_2$ affected [^{3}H]idazoxan binding in a dose-dependent manner with a maximal inhibition (75%) occurring at 150 mM. The half-maximal effective Mn^{2+} concentration was 40 mM.

In order to investigate the nature of the inhibition of [^{3}H]idazoxan binding by K^+ and Mn^{2+}, we performed saturation experiments in the presence of increasing concentrations of these cations. The addition of K^+ to the incubation mixture leads to a decrease in the binding affinity from 2.1 ± 0.2 to 4.1 ± 0.8 nM ($N = 3$, $P < 0.01$) at 40 mM and

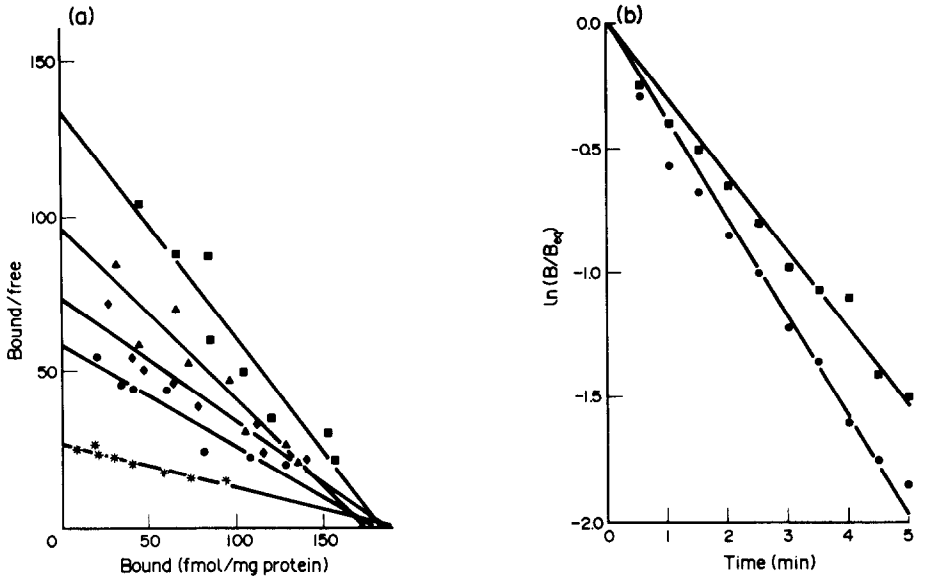

Fig. 5. Effect of KCl on [^{3}H]idazoxan binding to rabbit urethral membranes. (a) Saturation experiments with [^{3}H]idazoxan were performed in the absence (■) or in the presence of 10 mM (▲), 20 mM (♦), 40 mM (●) and 140 mM (*) KCl for 30 min at 28°. (b) Effect of KCl on the rate of [^{3}H]idazoxan dissociation. [^{3}H]Ligand (2.1 nM) was incubated for 30 min at 28°. Dissociation was initiated by addition of 25 μM phentolamine (■) or 25 μM phentolamine with 75 mM KCl (●). Samples were filtered at various times thereafter. Shown is the first order rate plot of decrease in specific binding with time. In this experiment the dissociation component increased from 0.26 min^{-1} (control) to 0.35 min^{-1} in the presence of 75 mM KCl.

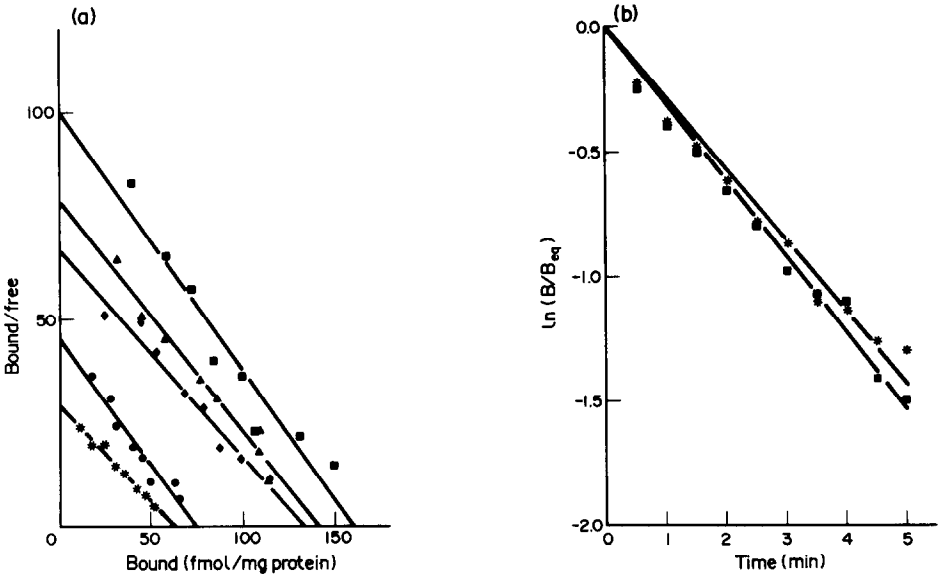

Fig. 6. Effect of $MnCl_2$ on [^{3}H]idazoxan binding to rabbit urethral membranes. (a) Saturation experiments with [^{3}H]idazoxan were performed in the absence (■) or in the presence of 10 mM (▲), 20 mM (♦), 50 mM (●) and 100 mM (*) $MnCl_2$ for 30 min at 28°. (b) Effect of $MnCl_2$ on the rate of [^{3}H]idazoxan dissociation. [^{3}H]Ligand (2.1 nM) was incubated for 30 min at 28°. Dissociation was initiated by addition of 25 μM phentolamine (■) or 25 μM phentolamine with 40 mM $MnCl_2$ (*). Samples were filtered at various times thereafter. Shown is the first order rate plot of decrease in specific binding with time. In this experiment the dissociation component was not modified (0.26 min^{-1} for the control and 0.25 min^{-1} in the presence of 40 mM $MnCl_2$).

7.0 ± 0.2 nM ($N = 3$, $P < 0.001$) at 140 mM KCl, without modification of maximal binding density (Fig. 5a).

In contrast, the addition of $MnCl_2$ decreases the binding density without change in binding affinity. The maximal number of binding sites was reduced from 197 ± 21 to 111 ± 19 fmol/mg protein ($N = 3$, $P < 0.05$) at 10 mM and 56 ± 10 fmol/mg protein ($N = 3$, $P < 0.001$) at 50 mM $MnCl_2$ (Fig. 6a). This decrease was maintained after extensive washing of $MnCl_2$ (40 mM) pretreated urethral membranes (not shown). In order to clarify the effects of these cations on [^{3}H]idazoxan binding we have monitored kinetics experiments in the presence of KCl (75 mM) or $MnCl_2$ (40 mM). The addition of these cations did not change significantly the association rate for [^{3}H]idazoxan (not shown).

In contrast, K^+ increased the dissociation rate from 0.257 ± 0.02 to 0.35 ± 0.02 min^{-1} ($P < 0.001$, $N = 5$) (Fig. 5b), while this constant was unchanged in the presence of Mn^{2+} (control: 0.26 ± 0.02; $MnCl_2$: 0.23 ± 0.03 min^{-1}, $N = 5$) (Fig. 6b).

The equilibrium dissociation constant (K_d) determined from the ratio k_{-1}/k_{+1} increased from 3.5 to 9.75 nM at 75 mM KCl while K_d was 2.77 nM at 40 mM $MnCl_2$. The ability of K^+ to accelerate the dissociation of [^{3}H]idazoxan from urethral membranes suggests that this cation binds to an allosteric site.

DISCUSSION

In rabbit urethral smooth muscle, [^{3}H]idazoxan binding is of high affinity, saturable, reversible and specific. These data provide additional evidence which suggests that these sites are not α_2-adrenoceptors.

We have previously proposed [13] that the [^{3}H]idazoxan binding sites in rabbit urethral smooth muscle could be an imidazoline receptor as initially demonstrated in bovine brainstem with [^{3}H]*p*-aminoclonidine [19]. However, from this study, competition experiments conducted with a large series of derivatives with an imidazoline ring show that imidazolidines such as clonidine and analogs inhibited [^{3}H]idazoxan binding with affinities which are in general lower than those observed for imidazoline compounds (cirazoline, naphazoline). These last compounds are differentiated from clonidine and analogs by the fact that they possess an aromatic ring linked to an unconstrained imidazoline group. In addition, it was previously reported that the imidazoline ring was not specifically required for the interaction with this urethral [^{3}H]idazoxan binding site. Guanidine derivatives, such as guanabenz, guanfacine, amiloride and its analogs [17], inhibited [^{3}H]idazoxan binding with affinities higher than those observed for clonidine or *p*-aminoclonidine.

This high affinity for guanidine analogs has not been reported for the imidazoline receptor previously characterized in bovine brain [23].

Moreover, as compared to the urethral [^{3}H]idazoxan binding site, bovine brain imidazoline receptor displays a sensitivity to histamine and cimetidine [19, 23]. Similar results with ours were recently reported in rabbit adipocytes [15] and rabbit kidney [18]. Taken together, these data show that pharmacological characteristics of the imidazoline binding sites differ considerably between species investigated and/or radioligand used. Conversely, as now established for α_2-adrenoceptors [24], subtypes of imidazoline binding sites may exist [25].

In contrast to that generally observed for α_2-adrenoceptors [9], [^{3}H]idazoxan binding to urethral smooth muscle membranes is highly sensitive to K^+ and Li^+, but weakly affected by Na^+. The half-maximal effective potassium concentration (60 mM) inhibiting ligand binding is more consistent with an effect mediated by intracellular rather than extracellular K^+. The addition of K^+ to the incubation mixture leads to a decrease in binding affinity without modification of maximal binding density. In addition, the ability of K^+ to accelerate the dissociation of [^{3}H]idazoxan from urethral membranes suggests that this cation binds to an allosteric site. Similar results were recently reported with K^+ in the rabbit renal proximal tubule [18].

Moreover, [^{3}H]idazoxan binding to urethral membranes is also specifically inhibited by Mn^{2+}. However, in contrast with results obtained with potassium ions, the addition of Mn^{2+} leads to a concentration-dependent decrease in binding capacity without change in affinity and dissociation kinetic. The inactivation of the [^{3}H]idazoxan binding sites by $MnCl_2$ appeared to be irreversible since there was no recovery after $MnCl_2$ pretreatment (40 mM) of the urethral membranes and extensive washing. The irreversibility of this phenomenon suggests that [^{3}H]idazoxan binding sites are altered by the addition of Mn^{2+}. This behaviour is unclear but it is conceivable that Mn^{2+} may interact with a membrane component susceptible to alter [^{3}H]idazoxan binding sites.

It has been previously shown that purified plasma membranes from human platelets contain α_2-adrenergic receptors but not the [^{3}H]idazoxan sites when compared to crude preparations [26]. In contrast, non-adrenergic [^{3}H]idazoxan binding sites have been observed on highly purified membranes of rabbit renal proximal tubules [18]. Taken together, these results suggest the possibility of one extra and intracellular distribution of [^{3}H]idazoxan sites. It is conceivable that an heterogeneity of [^{3}H]idazoxan sites could exist in crude urethral membranes. These sites could be differentiated by their sensitivity to Mn^{2+}.

As this Mn^{2+} effect has not been observed on highly purified renal membranes [18], the possibility is that this Mn^{2+} sensitive component may be lost during the renal preparation.

In conclusion, these data provide additional evidence that [^{3}H]idazoxan binding sites in rabbit urethral smooth muscle differ from α_2-adrenoceptors. In addition, their pharmacological properties appear markedly different from those reported for the imidazoline-preferring receptor in the central nervous system. Further investigations are needed to elucidate the physiological relevance of these sites in the rabbit urethral smooth muscle.

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