British Journal of Pharmacology (1997) 122, 43–50 

# Presynaptic imidazoline receptors and non-adrenoceptor [$^3$H]-idazoxan binding sites in human cardiovascular tissues

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**1** In segments of human right atrial appendages and pulmonary arteries preincubated with [$^3$H]-noradrenaline and superfused with physiological salt solution containing desipramine and corticosterone, the involvement of imidazoline receptors in the modulation of [$^3$H]-noradrenaline release was investigated.

**2** In human atrial appendages, the guanidines aganodine and DTG (1,3-di(2-tolyl)guanidine) which activate presynaptic imidazoline receptors, inhibited electrically-evoked [$^3$H]-noradrenaline release. The inhibition was not affected by blockade of $\alpha_2$-adrenoceptors with 1 $\mu$M rauwolscine, but antagonized by extremely high concentrations of this drug (10 and/or 30 $\mu$M; apparent $pA_2$ against aganodine and DTG: 5.55 and 5.21, respectively).

**3** In the presence of 1 $\mu$M rauwolscine, [$^3$H]-noradrenaline release in human atrial appendages was also inhibited by the imidazolines idazoxan and cirazoline, but not by agmatine and noradrenaline. The inhibitory effects of 100 $\mu$M idazoxan and 30 $\mu$M cirazoline were abolished by 30 $\mu$M rauwolscine.

**4** In the atrial appendages, the rank order of potency of all guanidines and imidazolines for their inhibitory effect on electrically-evoked [$^3$H]-noradrenaline release in the presence of 1 $\mu$M rauwolscine was: aganodine $\geqslant$ BDF 6143 [4-chloro-2-(2-imidazolin-2-yl-amino)-isoindoline] > DTG $\geqslant$ clonidine > cirazoline > idazoxan (BDF 6143 and clonidine were previously studied under identical conditions). This potency order corresponded to that previously determined at the presynaptic imidazoline receptors in the rabbit aorta.

**5** When, in the experiments in the human pulmonary artery, rauwolscine was absent from the superfusion fluid, the concentration-response curve for BDF 6143 (a mixed $\alpha_2$-adrenoceptor antagonist/imidazoline receptor agonist) for its facilitatory effect on electrically-evoked [$^3$H]-noradrenaline release was bell-shaped. In the presence of 1 $\mu$M rauwolscine, BDF 6143 and cirazoline concentration-dependently inhibited the evoked [$^3$H]-noradrenaline release.

**6** In human atrial appendages, non-adrenoceptor [$^3$H]-idazoxan binding sites were identified and characterized. The binding of [$^3$H]-idazoxan was specific, reversible, saturable and of high affinity ($K_D$: 25.5 nM). The specific binding of [$^3$H]-idazoxan (defined by cirazoline 0.1 mM) to membranes of human atrial appendages was concentration-dependently inhibited by several imidazolines and guanidines, but not by rauwolscine and agmatine. In most cases, the competition curves were best fitted to a two-site model.

**7** The rank order of affinity for the high affinity site (in a few cases for the only detectable site; cirazoline = idazoxan > BDF 6143 > DTG $\geqslant$ clonidine) is compatible with the pharmacological properties of $I_2$-imidazoline binding sites, but is clearly different from the rank order of potency for inhibiting evoked noradrenaline release from sympathetic nerves in the same tissue.

**8** It is concluded that noradrenaline release in the human atrium and, less well established, in the pulmonary artery is inhibited via presynaptic imidazoline receptors. These presynaptic imidazoline receptors appear to be related to those previously characterized in rabbit aorta and pulmonary artery, but differ clearly from $I_1$ and $I_2$ imidazoline binding sites.

**Keywords:** Imidazoline binding sites; presynaptic imidazoline receptors; human atrium; human pulmonary artery; [$^3$H]-idazoxan

## Introduction

It has been established that imidazoline derivatives and structurally related compounds such as guanidines bind not only to $\alpha_2$-adrenoceptors but also to imidazoline binding sites (IBS). The latter differ from the $\alpha_2$-adrenoceptors with respect to their structure, function and distribution among and within organs (for review, see e.g. Parini *et al*., 1996; Regunathan & Reis, 1996). Different rank orders of affinity of ligands for these sites indicate the existence of at least two classes of IBS denoted as $I_1$- and $I_2$-IBS. When $\alpha_2$-adrenoceptors are masked, [$^3$H]-clonidine is preferentially bound to $I_1$-IBS (e.g., Ernsberger *et al*., 1987; Molderings *et al*., 1993), whereas [$^3$H]-idazoxan labels $I_2$-IBS (e.g., Michel *et al*., 1989; Molderings *et al*., 1994). In addition, presynaptic imidazoline receptors mediating inhibition of noradrenaline release from postganglionic sympathetic nerve fibres have been identified in rabbit pulmonary artery, aorta and heart (Göthert & Molderings, 1991; Molderings *et al*., 1991; Fuder & Schwarz, 1993; Molderings & Göthert, 1995) which clearly differ from both $I_1$- and $I_2$-IBS.

Presynaptic imidazoline receptors are characterized by the following pharmacological properties which distinguish them from presynaptic $\alpha_2$-adrenoceptors (Starke, 1981; 1987). (1) Aganodine is a preferential imidazoline receptor agonist (Molderings *et al*., 1991; Fuder & Schwarz, 1993). (2) BDF 6143, a mixed $\alpha_2$-adrenoceptor antagonist/imidazoline receptor agonist, elicits a bell-shaped concentration-response curve for its facilitatory effect on evoked noradrenaline release.

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When the $\alpha_2$-autoreceptors are blocked by a non-imidazoline $\alpha_2$-adrenoceptor antagonist, the facilitatory effect of BDF 6143 is reversed to inhibition of evoked noradrenaline release (Göthert & Molderings, 1991; Molderings & Göthert, 1995). (3) The inhibitory effects of aganodine and BDF 6143 after blockade of $\alpha_2$-adrenoceptors are shared by the imidazoline derivative cirazoline, an $\alpha_1$-adrenoceptor agonist which is practically devoid of agonistic and antagonistic activity at $\alpha_2$-autoreceptors (Göthert & Molderings, 1991; Fink & Göthert, 1993; Molderings & Göthert, 1995). (4) After blockade of the $\alpha_2$-adrenoceptors, further imidazoline and guanidine derivatives such as clonidine, idazoxan and DTG, should inhibit the evoked noradrenaline release irrespective of whether or not they have affinity for $\alpha_2$-adrenoceptors, whereas the catecholamine noradrenaline does not (Göthert & Molderings, 1991; Molderings *et al*., 1991; Molderings & Göthert, 1995). (5) The $\alpha_2$-adrenoceptor antagonist rauwolscine acts as an antagonist at the presynaptic imidazoline receptors as well, but with markedly lower potency than at $\alpha_2$-adrenoceptors (Molderings *et al*., 1991; Fuder & Schwarz, 1993; Molderings & Göthert, 1995; Likungu *et al*., 1996).

Taking these criteria into account, the first aim of the present study was to characterize the presynaptic imidazoline receptors which have recently been found in human atrial appendages (Likungu *et al*., 1996) and to identify presynaptic imidazoline receptors on the sympathetic nerves of the human pulmonary artery. The second aim was to identify non-adrenoceptor [$^3$H]-idazoxan binding sites in the human atrial appendages. Finally, we examined whether the inhibitory potency of imidazolines at the presynaptic imidazoline receptors is related to their affinity for the non-adrenoceptor [$^3$H]-idazoxan binding sites, i.e. whether presynaptic imidazoline receptors exhibited similar pharmacological properties to these [$^3$H]-idazoxan binding sites.

Some of these data have been presented in a preliminary form (Molderings *et al*., 1996b; 1997).

## Methods

### *Functional experiments*

Segments of macroscopically normal human right atrial appendages and pulmonary arteries were obtained as leftovers from normotensive 35 to 70 year old male or female patients undergoing open heart or lung surgery, respectively. The atrial appendages were routinely removed for cannulation of the right atria. The patients were not treated with adrenoceptor agonists or antagonists, or with drugs influencing the storage or release of noradrenaline. After premedication with pethidine and promethazine, the patients were anaesthetized (both induction and maintenance) with flunitrazepam and fentanyl. During maintenance of anaesthesia they were ventilated with mixtures of oxygen and air. Pancuronium was administered for neuromuscular blockade. The study was approved by the local ethics committee.

The tissue segments were cut into strips of about $3 \times 15$ mm. These strips were incubated for 60 min in 1.5 ml physiological salt solution (37°C; composition see below) containing (−)-[ring-2,5,6-$^3$H]-noradrenaline 0.2 $\mu$M (specific activity 57.3 Ci mmol$^{-1}$). Subsequently, they were mounted vertically in an organ bath (tension adjusted to 2.0 g) between two parallel platinum electrodes and superfused with [$^3$H]-noradrenaline-free physiological salt solution, 37°C, at a rate of 2 ml min$^{-1}$. The composition of the solution was as follows (mM): NaCl 118, $NaH_2PO_4$, 1.2, $NaHCO_3$ 25, KCl 4.7, $CaCl_2$ 1.6, $MgSO_4$ 1.2, glucose 11.0, ascorbic acid 0.3, $Na_2EDTA$ 0.03 (aerated with 95% $O_2$ and 5% $CO_2$). Throughout the superfusion period, the solution contained desipramine 0.6 $\mu$M and corticosterone 40 $\mu$M, for blockade of neuronal and extraneuronal uptake of noradrenaline, respectively.

The superfusate was collected in 4-min fractions. Five (in a few experiments three or four) 3 min periods (2 Hz) of transmural electrical stimulation (rectangular pulses of 200 mA and 0.3 ms) were applied to each strip after 94 ($S_1$), 126 ($S_2$), 158 ($S_3$), 190 ($S_4$) and 222 ($S_5$) min of superfusion. At the end of superfusion the strips were solubilized with Soluene (Packard), and the radioactivity in the superfusate samples and blood vessels was determined by liquid scintillation counting.

The agonists under investigation were applied at concentrations increasing by a factor of 10 from 12 min before until 20 min after the onset of $S_3$, $S_4$ and $S_5$, respectively. The antagonists were present in the superfusion fluid from 14 min before $S_1$ until the end of the experiments. Separate control experiments were carried out for each series of experiments.

Tritium efflux was calculated as the fraction of tritium present in the strip at the onset of the respective collection period. Basal tritium efflux was expressed as the ratio of the fractional rate during the collection period immediately before $S_3$, $S_4$ or $S_5$ (i.e., $t_3$, $t_4$, $t_5$) over that immediately before $S_2$ ($t_2$). Stimulation-evoked tritium overflow was calculated by subtraction of the basal efflux from the total efflux during the 16 min subsequent to the onset of stimulation; basal efflux was assumed to decrease linearly from the collection period before to that 16–20 min after onset of stimulation. Evoked tritium overflow was calculated as a percentage of tissue tritium at the onset of stimulation, and the ratios of the overflow evoked by $S_3$, $S_4$ or $S_5$ over that evoked by $S_2$ were determined.

### *Radioligand binding experiments*

*Membrane preparation* All steps of the preparation procedure were performed on ice. The atrial segments were placed in 40 ml of a buffer solution containing HEPES-$Na^+$ 5 mM, EGTA 0.5 mM, $MgCl_2$ 0.5 mM, ascorbic acid 0.1 mM, PMSF 0.3 mM, pH 7.4 (buffer I), minced by means of an Ultraturrax ($5 \times 20$ s) and homogenized with a glass-Teflon homogenizer ($3 \times 30$ s). The homogenates were centrifuged (5 min, $1200 \times g$, 4°C). The supernatant was filtered through four layers of gauze, diluted to 420 ml with HEPES buffer I and recentrifuged (20 min, $40{,}000 \times g$, 4°C). The pellet was washed twice with buffer I, then resuspended in buffer II (HEPES-$Na^+$ 5 mM, EGTA 0.5 mM, $MgCl_2$ 0.5 mM, ascorbic acid 0.1 mM, pH 7.4), homogenized, diluted to give a protein concentration of about 2 mg ml$^{-1}$ and stored at −80°C until use. Before the membranes were added to the incubation assay, they were centrifuged (20 min, 40,000 *g*, 4°C), resuspended in the incubation buffer (buffer II), homogenized by ultrasonication and diluted to a final protein concentration of about 0.6 mg ml$^{-1}$.

*Binding assay* A 400 $\mu$l aliquot of membranes was incubated for 55 min with [$^3$H]-idazoxan (25 $\mu$l) at 4°C in a final volume of 0.5 ml. Saturation studies were performed with [$^3$H]-idazoxan 0.1–46 nM. Competition studies were done with [$^3$H]-idazoxan 10 nM and 13 different concentrations of the unlabelled ligand under investigation, ranging from 0.1 nM to 100 $\mu$M. Nonspecific binding was defined as [$^3$H]-idazoxan binding in the presence of cirazoline 100 $\mu$M, and accounted for 18% of the total radioactivity retained in the filters when [$^3$H]-idazoxan 10 nM was used. Adrenaline 10 $\mu$M, which has no affinity for imidazoline binding sites (Molderings *et al*., 1993; 1994) was added to the assay to prevent [$^3$H]-idazoxan from binding to $\alpha_2$-adrenoceptors. The reaction was stopped by rapid vacuum filtration with a Brandel cell harvester through Whatman GF/C glass fibre filters presoaked with polyethylenimine 0.5 M and clonidine 0.1 mM followed by rapid washing of the incubation tubes and filters with 10 ml ice-cold buffer II. Filters were placed in 6 ml scintillation fluid, shaken overnight and the radioactivity determined by liquid scintillation counting at 44% efficiency.

### *Data analysis and statistics*

Results from the functional experiments are given as means ± s.e.mean. Student's *t*-test was used for comparison of the mean values. $pIC_{30\%}$ values (negative logarithm of the con-

centration producing a 30% inhibition of evoked tritium overflow) was determined by interpolation from the nearest points of the concentration-response curves. Apparent $pA_2$ values were determined according to the following formula (Furchgott, 1972):

$$pA_2 = \log\left[\frac{[E']}{[E]}\right] - 1 - \log [B]$$

[E'] and [E] are the concentration of the agonists that caused 30% inhibition in the presence and absence of the antagonist, respectively. [B] is the concentration of the antagonist.

Data from the saturation and competition experiments were analysed by the least square fitting programme GraphPAD-inPlot (GraphPad Software Inc.). Results are expressed as mean values ± s.e.mean. All experiments were carried out in duplicate. For comparison of potencies of drugs in inhibiting [$^3$H]-noradrenaline release and their affinities for [$^3$H]-idazoxan binding sites, linear regression analysis was carried out.

### *Drugs used*

(−)-[ring-2,5,6-$^3$H]-noradrenaline (spec. activity 57.3 Ci mmol$^{-1}$; New England Nuclear, Dreieich, F.R.G); [$^3$H]-idazoxan (spec. activity 45 Ci mmol$^{-1}$; Amersham, U.K.); desipramine hydrochloride (Ciba-Geigy, Wehr, F.R.G.); agmatine sulphate, noradrenaline base, adrenaline base, corticosterone (Sigma, München, F.R.G.); (±)-idazoxan hydrochloride (Reckitt and Colman; Hull, U.K.); aganodine, 4-chloro-2-(2-imidazolin-2-ylamino)-isoindoline HCl (BDF 6143; Beiersdorf, Hamburg, F.R.G.); clonidine hydrochloride (Boehringer, Ingelheim, F.R.G.); rauwolscine hydrochloride (Roth, Karlsruhe, F.R.G.); 1,3-di(2-tolyl)guanidine (DTG; RBI, Natick, U.S.A.); cirazoline hydrochloride (Synthélabo, Paris, France). Drugs were dissolved in saline or water with the following exceptions: corticosterone was dissolved in 1,2-propandiol and the stock solution was further diluted with saline. Adrenaline was dissolved in HCl 0.01 M, DTG was dissolved in methanol; they were then further diluted in buffer II. The vehicles did not affect [$^3$H]-idazoxan binding.

## Results

### *Basal tritium efflux*

Under control conditions, basal tritium efflux from strips of atrial appendage and pulmonary artery preincubated with [$^3$H]-noradrenaline decreased with time, as reflected by the ratios $t_n/t_2$, which decreased from $t_3/t_2$ to $t_5/t_2$ (Table 1). Basal tritium efflux in the presence of 1, 10 and 30 μM rauwolscine did not significantly differ from that in the absence of this drug (data not shown). In the presence of 1 μM rauwolscine, 100 μM cirazoline increased basal tritium efflux in human atrial appendages by 56 ± 17% ($n = 8$); in human pulmonary artery 10 μM BDF 6143 increased basal tritium efflux by 42 ± 23% ($n = 5$). However, these moderate elevations of basal efflux did not exclude calculation of the electrically evoked tritium overflow at the respective stimulation period. By contrast, in the presence of 30 μM rauwolscine, basal tritium efflux was excessively increased by 100 μM cirazoline precluding the evaluation of the electrically-evoked tritium overflow. Therefore, we have tried to antagonize the effect of 30 μM cirazoline by 30 μM rauwolscine.

Lower concentrations of the drugs mentioned so far and of the other drugs at the concentrations investigated did not significantly affect the basal efflux of tritium (results not shown).

### *Electrically-evoked tritium overflow in control experiments*

Transmural electrical stimulation was applied to strips of atrial appendages or pulmonary artery at 2 Hz for 3 min.

In experiments on strips of atrial appendages, which were carried out in the presence of 1 μM rauwolscine to block presynaptic $\alpha_2$-adrenoceptors, the tritium overflow evoked by $S_2$ tended to be increased by rauwolscine (present from 14 min before $S_1$ until the end of the experiments; Table 1) and a similar result was obtained with 10 and 30 μM rauwolscine (results not shown).

**Table 1** Control values for basal tritium efflux and stimulation-evoked tritium overflow from strips of human atrial appendages and pulmonary arteries preincubated with [$^3$H]-noradrenaline and superfused with [$^3$H]-noradrenaline-free solution containing 0.6 μM desipramine plus 40 μM corticosterone. Five 3 min periods of transmural electrical stimulation (2 Hz; $S_1$, $S_2$, $S_3$, $S_4$ and $S_5$) were applied to each preparation after 94 ($S_1$), 126 ($S_2$), 158 ($S_3$), 190 ($S_4$) and 222 min ($S_5$) of superfusion. Means ± s.e.mean of 5–7 experiments are shown

| | *Basal $^3$H efflux*[a,b] | | | | |
|---|---|---|---|---|---|
| *Experimental condition* | $t_2$ (nCi min$^{-1}$) | $t_2$ fractional rate (min$^{-1}$)[c] | $t_3/t_2$ | $t_4/t_2$ | $t_5/t_2$ |
| *Atrial appendages* | | | | | |
| No rauwolscine | 2.7 ± 0.4 | 0.0007 ± 0.0001 | 0.86 ± 0.02 | 0.80 ± 0.02 | 0.77 ± 0.04 |
| Rauwolscine 1 μM[d] | 2.4 ± 0.5 | 0.0007 ± 0.0001 | 0.89 ± 0.02 | 0.80 ± 0.02 | 0.76 ± 0.04 |
| *Pulmonary artery* | | | | | |
| No rauwolscine | 0.9 ± 0.2 | 0.0006 ± 0.0001 | 0.86 ± 0.01 | 0.78 ± 0.02 | 0.72 ± 0.02 |
| Rauwolscine 1 μM[d] | 0.9 ± 0.1 | 0.0007 ± 0.0001 | 0.89 ± 0.03 | 0.80 ± 0.05 | 0.80 ± 0.04 |
| | *Evoked $^3$H overflow*[b] | | | | |
| *Experimental condition* | $S_2$ (nCi) | $S_2$ (% of tissue tritium)[e] | $S_3/S_2$ | $S_4/S_2$ | $S_5/S_2$ |
| *Atrial appendages* | | | | | |
| No rauwolscine | 17.2 ± 3.9 | 1.01 ± 0.24 | 1.04 ± 0.02 | 1.02 ± 0.05 | 0.89 ± 0.04 |
| Rauwolscine 1 μM[d] | 23.5 ± 6.7 | 1.70 ± 0.41 | 0.91 ± 0.09 | 0.85 ± 0.09 | 0.72 ± 0.07 |
| *Pulmonary artery* | | | | | |
| No rauwolscine | 3.1 ± 0.5 | 0.82 ± 0.12 | 0.97 ± 0.04 | 0.92 ± 0.03 | 0.91 ± 0.04 |
| Rauwolscine 1 μM[d] | 9.6 ± 2.0 | 2.3 ± 0.55 | 0.91 ± 0.06 | 1.00 ± 0.07 | 0.86 ± 0.04 |

[a]$t_1 - t_5$ represent the 4 min periods of superfusate sampling immediately before the respective stimulation periods ($S_1 - S_5$). [b]Basal efflux during $t_3$, $t_4$ and $t_5$ and overflow evoked by $S_3$, $S_4$ and $S_5$ are given as ratios over the respective $t_2$ and $S_2$ value. [c]Efflux of tritium min$^{-1}$, expressed as fraction of tissue tritium. [d]Rauwolscine was present in the superfusion fluid from 14 min before $S_1$ until the end of the experiments. [e]Evoked tritium overflow above basal efflux, expressed as percentage of tissue tritium.

In experiments with 1 $\mu$M rauwolscine on strips of pulmonary artery, the tritium overflow evoked by $S_2$ was three times higher than in the experiments without rauwolscine, indicating a tonic activation of the $\alpha_2$-autoreceptors by endogenous noradrenaline (Table 1).

Under all of these conditions, the evoked overflow either slightly decreased from $S_2$ to $S_5$ or remained approximately constant, as reflected by the $S_n/S_2$ ratios (Table 1).

### *Effects of drugs on electrically-evoked tritium overflow from atrial appendages*

Noradrenaline and the two guanidine derivatives aganodine and DTG inhibited the electrically (2 Hz)-evoked tritium overflow in the absence of rauwolscine ($pIC_{50\%}$ values: 7.54, 6.30 and 5.83, respectively; Figure 1, open symbols). Rauwolscine 1 $\mu$M abolished the noradrenaline-induced inhibition of tritium overflow, but failed to influence the concentration-response curves for aganodine and DTG (Figure 1; for $IC_{30\%}$, see Table 2). Rauwolscine 10 and 30 $\mu$M shifted the concentration-response curve of aganodine to the right yielding apparent $pA_2$ values of 5.45 and 5.64, respectively (determined at the level of the $IC_{30\%}$ values of aganodine). Rauwolscine 30 $\mu$M shifted the concentration-response curve of DTG to the right yielding an apparent $pA_2$ value of 5.21 (determined at the level of the $IC_{30\%}$ values of DTG).

In the presence of 1 $\mu$M rauwolscine, the imidazolines cirazoline and idazoxan resembled aganodine and DTG in that they inhibited the electrically-evoked tritium overflow, although at lower potency (Figure 2; for $IC_{30\%}$, see Table 2). In contrast, agmatine, an endogenous guanidine with moderate affinity for imidazoline binding sites (Li *et al*., 1994), did not inhibit evoked tritium overflow under these conditions (Figure 2). Rauwolscine at the concentration of 30 $\mu$M abolished the inhibitory effects of 30 $\mu$M cirazoline and 100 $\mu$M idazoxan (Figure 2).

### *Effects of drugs on electrically-evoked tritium overflow from pulmonary artery*

At the stimulation frequency of 2 Hz, BDF 6143 induced a biphasic increase in electrically-evoked tritium overflow. Up to 0.1 $\mu$M, the BDF 6143-induced facilitation increased with the concentration applied, whereas this effect was less pronounced at 1 $\mu$M BDF 6143 than at a 10 times lower concentration (Figure 3; open symbols). In the presence of 1 $\mu$M rauwolscine, BDF 6143 concentration-dependently inhibited the electrically-evoked tritium overflow (Figure 3; for $IC_{30\%}$, see Table 2). Under this condition, cirazoline also inhibited the evoked tritium overflow (Figure 3; for $IC_{30\%}$, see Table 2).

### *[$^3$H]-idazoxan binding*

Figure 4 shows the equilibrium specific binding of [$^3$H]-idazoxan to human atrial membranes as a function of the radioligand concentration. The best-fitting equation obtained by non-linear regression analysis describes the reaction of [$^3$H]-idazoxan with one binding site with a $K_D$ value of $25.5 \pm 3.8$ nM and a $B_{max}$ of $425 \pm 60$ fmol mg$^{-1}$ protein ($n = 4$). A low affinity binding component (see below) could not be detected in the radioligand concentration range applied.

In competition experiments, most of the compounds listed in Table 3 concentration-dependently inhibited specific binding of [$^3$H]-idazoxan 10 nM (Figure 5); at this radioligand concentration, specific binding amounted to $2742 \pm 146$ d.p.m. (corresponding to $82 \pm 2\%$ of total binding; $n = 34$). A total or nearly total inhibition was obtained with cirazoline, BDF 6143, idazoxan, aganodine and DTG, in the concentration range investigated (up to 100 $\mu$M; Figure 5; Table 3). Clonidine 100 $\mu$M inhibited the specific [$^3$H]-idazoxan binding by 43% only. Agmatine and rauwolscine at concentrations up to 100 $\mu$M did not inhibit specific binding of [$^3$H]-idazoxan (not shown).

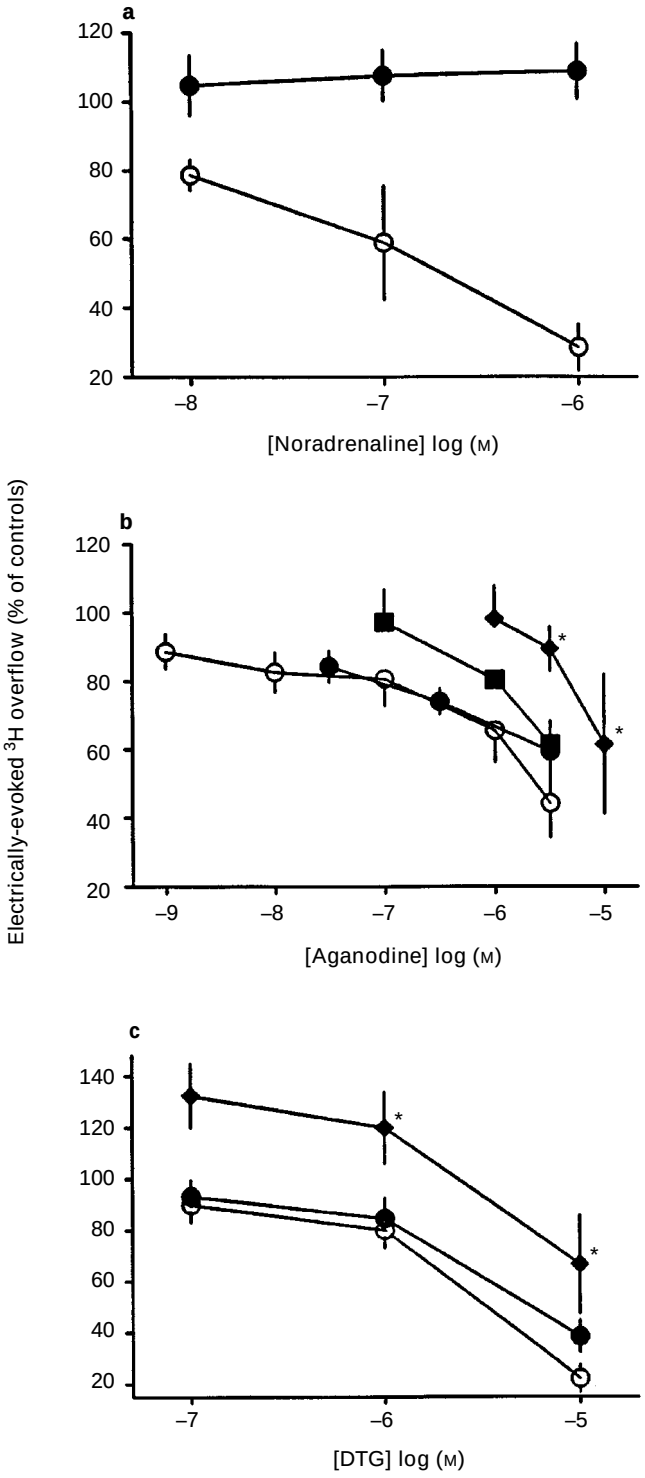


**Figure 1** Effects of (a) noradrenaline and of the guanidine derivatives (b) aganodine and (c) DTG on electrically evoked tritium overflow from segments of human atrial appendages and interaction with rauwolscine. Experiments without rauwolscine (open symbols); experiments in the presence of rauwolscine 1 $\mu$M (●), 10 $\mu$M (■) and 30 $\mu$M (◆) from 14 min before $S_1$ until the end of the experiments. Ordinate scales, $S_3/S_2$, $S_4/S_2$ overflow ratios, expressed as percentage of ratios in respective control experiments without noradrenaline, aganodine or DTG administration. Means from 4–12 tissue strips; vertical lines show s.e.mean. All changes in evoked tritium overflow that exceeded 20% (compared with the corresponding controls) were statistically significant (at least $P < 0.05$); two exceptions referred to 10 $\mu$M aganodine and 10 $\mu$M DTG in the presence of 30 $\mu$M rauwolscine which did not produce a significant inhibition. *$P < 0.05$ (compared with the effect of the same aganodine and DTG concentration, respectively, in the absence of rauwolscine).

**Table 2** Potency ($pIC_{30\%}$ values) of imidazolines, guanidines and of noradrenaline in inhibiting the electrically (2 Hz)-evoked tritium overflow from strips of human atrial appendages and pulmonary artery in the presence of 1 $\mu$M rauwolscine

| *Compound* | *Human atrial appendages* | *Human pulmonary artery* |
|---|---|---|
| Aganodine | 6.34 | ND |
| BDF 6143 | 6.20[a] | 6.70 |
| DTG | 5.68 | ND |
| Clonidine | 5.50[a] | ND |
| Cirazoline | 4.44 | 5.35 |
| Idazoxan | 4.33 | ND |
| Noradrenaline | < 6 | ND |

[a]Data were taken from Likungu *et al.* (1996). ND not determined.

Competition of BDF 6143, idazoxan, aganodine, clonidine and DTG with [$^3$H]-idazoxan 10 nM revealed inhibition curves with a slope factor of less than 1. Hence, these curves were significantly better fitted to a two-site than to a one-site model (Figure 5; Table 3). Cirazoline revealed a monophasic displacement curve ($n_H$ not significantly different from 1.0). The $K_i$ values at the high-affinity or the single site for the drugs listed in Table 3 ranged from 7 to 1440 nM with a rank order of affinities as follows (Table 3): cirazoline = idazoxan > aganodine > BDF 6143 > DTG ≥ clonidine.

### *Correlations*

Comparison of the potencies of the imidazolines and guanidines in inhibiting the electrically-evoked tritium overflow from human atrial appendages, with the affinities of the compounds for the high affinity non-adrenoceptor [$^3$H]-idazoxan binding sites in the same tissue, revealed no significant correlation (Figure 6).

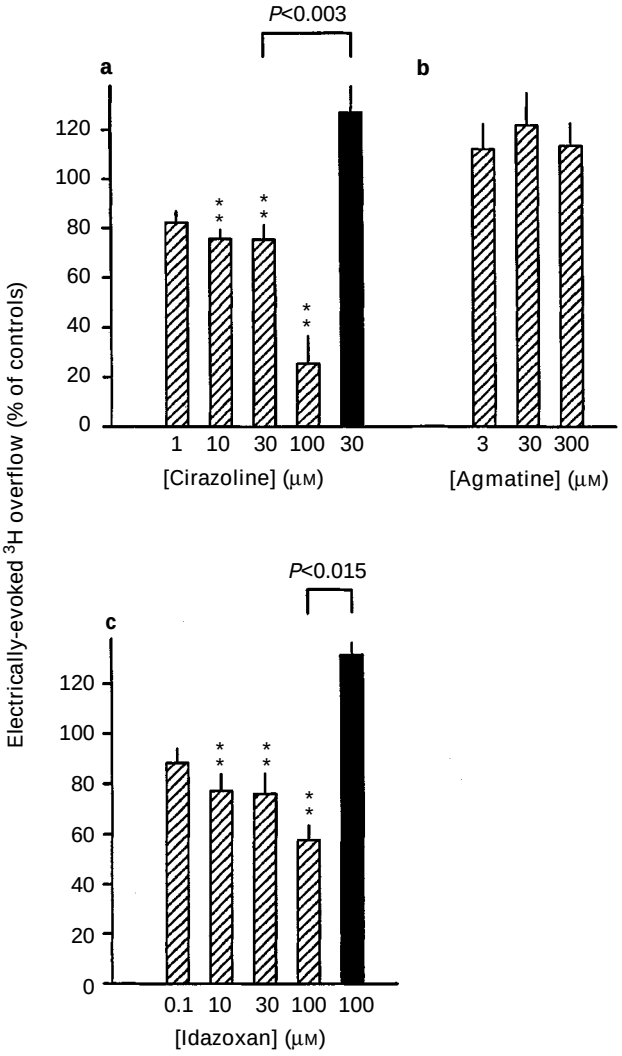


**Figure 2** Effects of (a) cirazoline, (c) idazoxan and (b) agmatine on electrically-evoked (2 Hz) tritium overflow from segments of human atrial appendages in the presence of 1 or 30 $\mu$M rauwolscine (hatched and solid columns, respectively). Rauwolscine was present from 14 min before $S_1$ until the end of the experiments. Ordinate scales, $S_3/S_2$, $S_4/S_2$ and $S_5/S_2$ overflow ratios, expressed as percentage of ratios in respective control experiments without agonist administration. Means ± s.e.mean from ($n$) tissue strips. Cirazoline (4–13), idazoxan (5–11), agmatine (6). $^{**}P<0.01$ (compared with the corresponding controls).

## Discussion

### *Functional experiments*

The initial aim of the present study was to determine the pharmacological properties of the presynaptic imidazoline receptors which have recently been identified in human atrial appendages (Likungu *et al.*, 1996) and to provide hints for the existence of such inhibitory presynaptic imidazoline receptors on the sympathetic nerves of the human pulmonary artery as well (a tissue which is no longer available to us). The electrically-evoked tritium overflow from superfused segments of atrial appendages and pulmonary artery preincubated with [$^3$H]-noradrenaline was determined, which under the present conditions (blockade of neuronal and extraneuronal uptake) reflects the action potential-induced release of tritiated and endogenous noradrenaline from the sympathetic neurones (for details, see Molderings *et al.*, 1996a).

*Human atrial appendages* The present results confirm the existence of presynaptic imidazoline receptors in human atrial appendages and extend the previous findings obtained with clonidine and moxonidine (Likungu *et al.*, 1996), inasmuch as the pharmacological properties of these receptors have been determined in detail. All of the criteria of presynaptic imidazoline receptors defined and listed in the Introduction have been established for the atrial presynaptic imidazoline receptors in the present or, to a minor part, in our previous study (Likungu *et al.*, 1996).

(1) In the presence of rauwolscine at a concentration which abolished the inhibitory effect of noradrenaline, aganodine proved to be most potent in inhibiting noradrenaline release, i.e. in activating the imidazoline receptors.

(2) In the presence of the non-imidazoline $\alpha_2$-adrenoceptor antagonist rauwolscine, the mixed $\alpha_2$-adrenoceptor antagonist/imidazoline receptor agonist BDF 6143 acted as a pure inhibitor of noradrenaline release (Likungu *et al.*, 1996). In the absence of rauwolscine, there was only a tendency towards a facilitation of noradrenaline release since the $\alpha_2$-autoreceptors in this tissue (Rump *et al.*, 1995a,b; Likungu *et al.*, 1996) appear not to be strongly activated by endogenous noradrenaline under physiological conditions. A conceivable explanation for this finding is that the synaptic cleft in the human atrium may be relatively wide, leading to a low concentration of noradrenaline in the biophase of the $\alpha_2$-autoreceptors. In agreement with this assumption, interruption of the autoinhibitory feedback loop by rauwolscine led only to an inconsistent increase in noradrenaline release which was not statistically significant (this study).

(3) The finding that cirazoline in the presence of rauwolscine inhibits noradrenaline release (this study) is compatible with

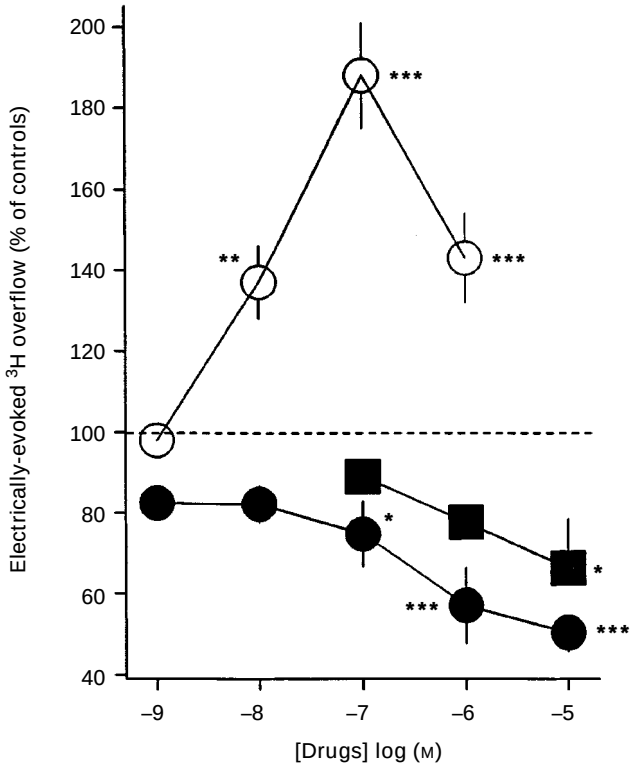


**Figure 3** Effects of BDF 6143 (○, ●) and cirazoline (■) on electrically-evoked (2 Hz) tritium overflow from segments of human pulmonary artery in the absence (open symbols) and presence of 1 $\mu$M rauwolscine (solid symbols; from 14 min before $S_1$ until the end of the experiments). Ordinate scales, $S_3/S_2$, $S_4/S_2$ and $S_5/S_2$ overflow ratios, expressed as percentage of ratios in respective control experiments without BDF 6143 or cirazoline administration. Means from 4–10 tissue strips; vertical lines show s.e.mean. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (compared with the corresponding controls).

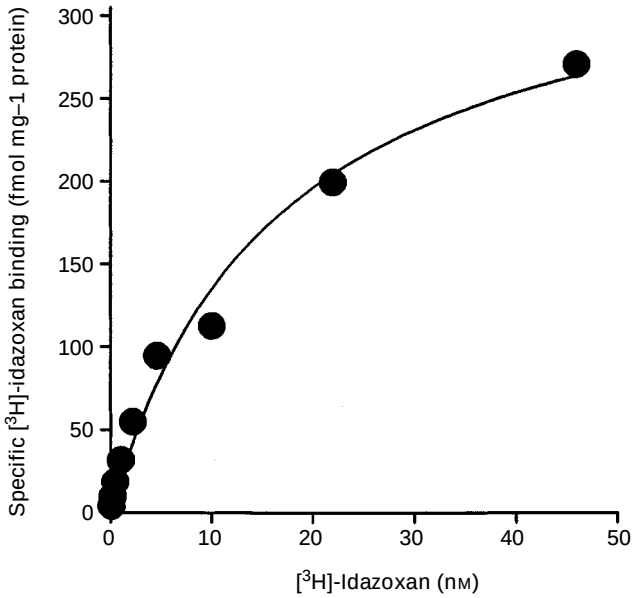


**Figure 4** Saturation curve for specific [$^3$H]-idazoxan binding. Membranes from human atrial appendages were incubated for 55 min at 4°C with increasing concentrations of [$^3$H]-idazoxan. The graph shows one representative experiment out of 4 performed in duplicate.

**Table 3** Potencies ($K_i$ values) of imidazolines, guanidines and rauwolscine in inhibiting specific [$^3$H]-idazoxan binding from its high and low affinity binding sites (high and low) in membranes from human atrial appendages. Results from computer analysis of competition curves[a] obtained by adding various concentrations of a competing ligand and a fixed concentration (10 nM) of [$^3$H]-idazoxan (see Methods for details). The percentages of high ($\%_{high}$) and low ($\%_{low}$) affinity sites are given for drugs with competition curves which were best resolved (partial *F*-test, last column) in a two-site-fit. With each compound, 4–6 experiments were performed in duplicate

| *Compound* | *High* (nM) | $\%_{high}$ | *Low* (nM) | $\%_{low}$ | *Partial* F-*test* |
|---|---|---|---|---|---|
| Cirazoline | 6.6 | 100 | | | |
| Idazoxan | 6.8 | 74 | 641 | 26 | $P<0.006$ |
| Aganodine | 33 | 47 | 12375 | 53 | $P<0.002$ |
| BDF 6141 | 86 | 82 | 7127 | 18 | $P<0.008$ |
| DTG | 1152 | 19 | 640700 | 81 | $P<0.03$ |
| Clonidine | 1440 | 21 | 158375 | 79 | $P<0.02$ |
| Agmatine | >100000 | | | | |
| Rauwolscine | >100000 | | | | |

[a]A logistic function was fitted to average competition data to obtain slope factors (Hill coefficients, $n_H$ and $IC_{50}$ values). $K_i$ values were then calculated from the $IC_{50}$ values, the $K_D$ value of [$^3$H]-idazoxan and the [$^3$H]-idazoxan concentration used (Cheng & Prusoff, 1973).

the involvement of imidazoline receptors and argues against the possibility that an $\alpha_2$-adrenoceptor is involved. Cirazoline is an agonist at $\alpha_1$-adrenoceptors (see, e.g. Ruffolo & Waddell, 1982) which are not present on the sympathetic nerves of the human right atrium (Rump *et al*., 1995b).

(4) In the presence of rauwolscine at a concentration which abolished the noradrenaline-induced inhibition, clonidine (Likungu *et al*., 1996), idazoxan and DTG (a guanidine derivative with negligible affinity for $\alpha_2$-adrenoceptors; Weber *et al*., 1986) shared the ability of aganodine, BDF 6143 and cirazoline to inhibit noradrenaline release in human atria (present study).

(5) At extremely high concentrations, rauwolscine antagonized the inhibitory effect of the imidazoline receptor agonists aganodine, DTG, idazoxan and cirazoline (this study). This result is basically compatible with previous findings in the rabbit pulmonary artery and aorta in which rauwolscine also acted as a low potency antagonist at the presynaptic imidazoline receptors (apparant $pA_2$ against aganodine and cirazoline in the range of 6.67–7.32, respectively; Molderings *et al*., 1991; Molderings & Göthert, 1995). It should be noted that, in the human atrial appendages, the potency of rauwolscine as an antagonist at presynaptic imidazoline receptors was even 1–2 log units lower (mean apparent $pA_2$ against aganodine: 5.55; apparent $pA_2$ against DTG: 5.21).

In spite of the quantitative difference in the antagonistic potency of rauwolscine at the presynaptic imidazoline receptors between the human atrial appendages and rabbit blood vessels, the basic similarity in the action of rauwolscine in both species conforms to the idea that the presynaptic imidazoline receptors in the cardiovascular tissue of both species are homologous. This suggestion is supported by two additional findings. Firstly, the potencies ($pIC_{30\%}$ values) of the imidazolines and guanidines in inhibiting noradrenaline release in the human atrium (Table 2) significantly correlated with their potencies previously determined under similar conditions in the rabbit aorta (Molderings & Göthert, 1995; $r=0.94$, $P<0.006$). Again, there was a quantitative difference between both species, the agonists being 0.15–1.01 log unit less potent in human cardiac tissue than in the rabbit blood vessels. Secondly, as a further similarity between human and rabbit presynaptic imidazoline receptors, the imidazoline derivative moxonidine which exhibits high affinity for $I_1$-IBS (Ernsberger *et al*., 1992) failed

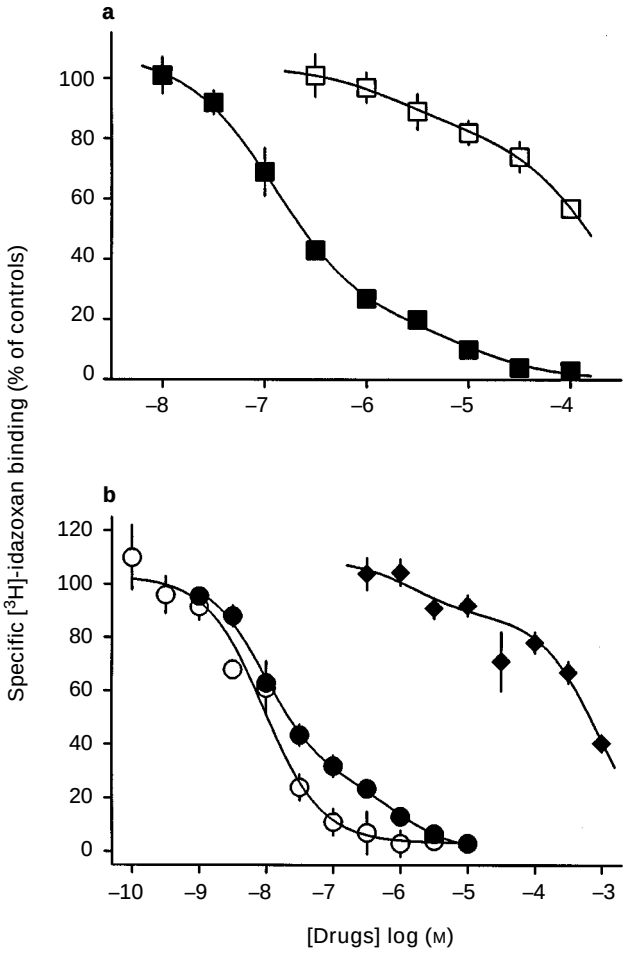


**Figure 5** Competition of four imidazoline derivatives (a) clonidine (□), BDF 6143 (■), (b) cirazoline (○), idazoxan (●) and a guanidine derivative (DTG, ◆) with [$^3$H]-idazoxan for its specific binding sites in human atrial membranes. Membranes were incubated for 55 min with [$^3$H]-idazoxan 10 nM and increasing concentrations of the respective competitor. Each point is the mean of 4–6 experiments performed in duplicate; vertical lines show s.e.mean.

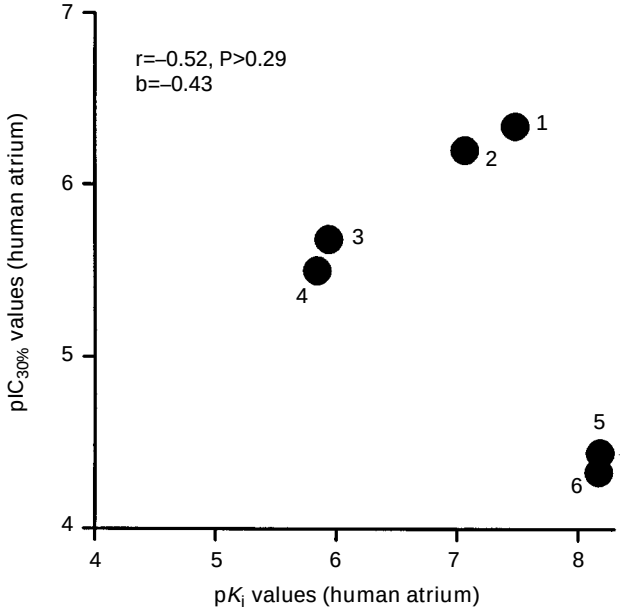


**Figure 6** Comparison of the potencies ($pIC_{30\%}$ values) of imidazolines and guanidines in inhibiting the electrically-evoked tritium overflow in human atrial appendages with their affinities ($pK_i$ values) for the high-affinity non-adrenoceptor [$^3$H]-idazoxan binding sites in human atrium. *r*: correlation coefficient; *P*: level of significance; b: slope of the regression line. (1) Aganodine, (2) BDF 6143, (3) DTG, (4) clonidine, (5) cirazoline, (6) idazoxan.

to stimulate the presynaptic imidazoline receptors not only in the human atrium (Likungu *et al*., 1996) but also in the rabbit pulmonary artery (Molderings *et al*., 1991) and aorta (Molderings & Göthert, 1995).

*Human pulmonary artery* The human pulmonary artery resembled the rabbit aorta and pulmonary artery (Göthert & Molderings, 1991; Molderings *et al*., 1991; Molderings & Göthert, 1995) in that BDF 6143 induced a bell-shaped concentration-facilitation response curve. The facilitatory effect of BDF 6143 was probably due to its antagonistic effect at $\alpha_2$-autoreceptors, which, at the stimulation frequency of 2 Hz, are tonically activated by endegonous noradrenaline (see $S_2$ values in the absence and presence of rauwolscine in Table 1). After blockade of the $\alpha_2$-autoreceptors by rauwolscine, BDF 6143 acted as a pure inhibitor of the electrically-evoked noradrenaline release at a potency which was very similar to that in the rabbit aorta (Molderings & Göthert, 1995). The same held true for the potency of cirazoline in inhibiting noradrenaline release when the $\alpha_2$-adrenoceptors were blocked by rauwolscine. Thus, two of the pharmacological criteria for the existence of presynaptic imidazoline receptors listed in the Introduction are fulfilled, suggesting that the human pulmonary artery seems to be also endowed with inhibitory presynaptic imidazoline receptors. Due to the lack of possibility to obtain further experimental support (see above), this conclusion should be drawn with caution, but any other interpretation would be less plausible.

## *[$^3$H]-idazoxan binding*

The second aim of the present study was to examine whether [$^3$H]-idazoxan binds with high affinity to specific non-adrenoceptor binding sites in human atrial tissue. Under our experimental conditions, binding of [$^3$H]-idazoxan was specific, saturable and of high affinity. These features fulfill the criteria for the identification of a specific recognition site. In the saturation experiments, only one [$^3$H]-idazoxan binding site was identified, whereas in competition experiments with BDF 6143, idazoxan, aganodine, clonidine, and DTG shallow displacement curves ($n_H < 1.0$) were obtained which were fitted best to two binding sites by computer modelling. This discrepancy is probably due to the fact that in the saturation experiments [$^3$H]-idazoxan was applied at concentrations of up to 46 nM only, far too low for the identification of the low affinity site, while ligand concentrations of up to at least 10 $\mu$M were investigated in the competition experiments. The $K_D$ value estimated from the saturation experiments (26 nM) was in a similar range as the $K_i$ value obtained in the competition studies (7 nM) for the high affinity site and as the $K_D$ values reported in the literature for non-adrenoceptor [$^3$H]-idazoxan binding sites (11 nM, Wikberg *et al*., 1992; 13 nM, Molderings *et al*., 1994). Shallow inhibition curves with Hill coefficients significantly different from unity have previously been found in several studies on imidazoline binding sites, e.g. in guinea-pig kidney (Wikberg *et al*., 1992) and in bovine chromaffin cells (Molderings *et al*., 1994; 1995). On the basis of these findings, it has been suggested that IBS may exist in two interconvertible forms which are induced by the ligands at different proportions (Wikberg *et al*., 1992; Li *et al*., 1994).

For the following reasons our data exclude the possibility that, under the present experimental conditions, [$^3$H]-idazoxan labels $\alpha_2$-adrenoceptors: (1) (−)-adrenaline (100 $\mu$M) was added to each assay tube in order to protect the radioligand from

binding to $\alpha_2$-adrenoceptors. (2) In the competition experiments, the $\alpha_2$-adrenoceptor antagonist rauwolscine exhibited no affinity for the [$^3$H]-idazoxan binding sites. (3) The rank order of potency of all $\alpha_2$-adrenoceptor ligands in displacing [$^3$H]-idazoxan (Table 3) differed from the rank order expected for binding to the various $\alpha_2$-adrenoceptor subtypes (for review, see e.g. Bylund *et al*., 1994).

The rank order of affinity of the competing drugs for the [$^3$H]-idazoxan binding sites in human atria (Table 3) was clearly different from that previously found for $I_1$-IBS in bovine adrenal medulla (i.e. clonidine = cirazoline > BDF 6143 > idazoxan; Molderings *et al*., 1993). This finding excludes the possibility that the [$^3$H]-idazoxan binding site represents an $I_1$-IBS. In contrast, the rank order of affinity of the competing imidazolines found in the present experiments conforms to their rank order at $I_2$-IBS in bovine adrenal medulla (i.e. cirazoline $\geqslant$ idazoxan > BDF 6143 > clonidine; Molderings *et al*., 1994). Accordingly, the IBS identified here may be denoted as $I_2$-IBS.

Finally and most importanty, the possibility had to be considered that the $I_2$-IBS and the presynaptic imidazoline receptors in human atrium might be identical, but potencies determined in the release experiments did not correlate with the affinities in the radioligand binding experiments (Figure 6). Thus, the possibility that the presynaptic imidazoline receptor in human atrial appendages represents an $I_2$-receptor was excluded.

The technical assistance of Mrs D. Funccius and Mrs M. Hartwig is gratefully acknowledged. This study was supported by a grant of the Deutsche Forschungsgemeinschaft.

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*(Received March 13, 1997*
*Revised May 21, 1997*
*Accepted May 30, 1997)*