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Characterization of α_2-adrenoceptor binding properties of imidazoline-like drugs, azoloazepine derivatives and β-phenethylamine-like drugs in human platelet membranes

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To characterize the agonist profile of α_2-adrenoceptor agonists (imidazoline-like drugs, azoloazepine derivatives, β-phenethylamines-like drugs) on human platelets, the characteristics of α_2-adrenoceptors (K_D, B_{max}) have been evaluated and the affinity constants measured by displacement technique and computer-assisted analysis of the curves. Furthermore, since α_2-adrenoceptor agonists interact with the post-synaptic receptors in a calcium-operated channel, whether the effect of calcium-entry inhibitors (verapamil, nifedipine, diltiazem) is related to a competition with α_2-receptors has also been examined. By Scatchard analysis, it was calculated that in human platelets α_2-adrenoceptors have K_D = 3·45 nM and B_{max} = 247 fmol (mg protein)$^{-1}$. As far as the potency is concerned, imidazoline-like drugs were the most potent agonists in human platelet α_2-adrenoceptors (guanabenz IC50 = 8·6 ± 0·8 × 10^{-8}, B-HT 920 IC50 = 2·9 ± 0·3 × 10^{-7}, (−)-adrenaline IC50 = 3·4 ± 0·5 × 10^{-7}). Among the calcium-entry inhibitors only verapamil antagonized [^{3}H]rauwolscine binding: the effect was stereospecific, (−)-D 600 being more potent than (+)-D 600. Nifedipine and diltiazem did not affect α_2-receptor binding. It is concluded that human platelets α_2-receptors share the agonist potency profile of other tissues containing α_2-receptors (brain, pre-synaptic junction), and that among calcium-entry blockers only verapamil can antagonize α_2-agonists. Nifedipine and diltiazem do not appear to interact stereospecifically with α_2-adrenoceptors.

The original subdivision of α-adrenoceptors on anatomical and functional grounds (post-synaptic: α_1; pre-synaptic: α_2) (Berthelson & Pettinger 1977; Langer 1980) has not received support from recent data obtained by radioligand binding studies (Hoffmann & Lefkowitz 1980) and by animal studies (pithed rat) (Timmermans & van Zwieten 1980). It is known that α_2-adrenoceptors are not exclusively located pre-synaptically and there is ample evidence for the existence of α_2-adrenoceptors outside noradrenergic terminal axons and at post-synaptic sites (Timmermans & van Zwieten 1980). Post-synaptic α_2-adrenoceptors have been identified in vascular smooth muscle of various animal species, including rat (Drew & Whiting 1979), rabbit (van Meel et al 1982), dog (Constantine et al 1980a, b), cat (Timmermans & van Zwieten 1981) and man (Jie et al 1984). These results have been achieved by the availability of highly specific agonists B-HT 933 (Kobinger & Pichler 1977) and B-HT 920 (Kobinger et al 1980) which increase vascular smooth muscle contractility by acting on vascular α_2-adrenoceptors. Furthermore, studies on pre-synaptic α-receptors, in rabbit hypothalamic cells, have shown that verapamil selectively enhances [^{3}H]noradrenaline efflux with an antagonizing interaction (Galzin & Langer 1983).

Since human platelets contain α_2-adrenoceptors, well characterized as far as the number and the affinity constants are concerned (Hoffmann et al 1979), we aimed at a comprehensive agonist potency profile of these receptors. Therefore we evaluated the displacement curves of the main groups of α_2-adrenoceptor agonists, i.e. the β-phenethylamine-like drugs, the imidazoline-like drugs and the azoloazepine derivatives (Reid et al 1983; Timmermans & van Zwieten 1982).

We also investigated the binding profile of verapamil and its methoxyderivatives (+)- and (−)-D 600 (Fleckenstein 1977). We also studied how stereospecificity affected the antagonism on α_2-platelet adrenoceptors of nifedipine and diltiazem, two calcium-blockers that antagonize the pressor effects of azoloazepines on vascular smooth muscle α_2-adrenoceptors (van Zwieten et al 1983a, b).

Materials and methods

Human platelets were obtained on the morning of the experiment by venepuncture from normal blood donors (n = 40: 32 males, 8 females; age range: 32–48 years); blood (250 ml) was drawn and acid-citrate-dextrose was used as anticoagulant (ACD). A platelet-rich plasma was prepared by centrifuging at 160*g* for 15 min at 25 °C; the platelet-rich plasma was then diluted with the 'washing buffer' (Tris-HCl 0·05 M, NaCl 0·15 M EDTA 0·02 M, pH 7·5) and centrifuged at 5100*g* for 15 min at 25 °C. The washed pellet was homogenized in 'hypotonic buffer' (Tris-HCl 5 mM. EDTA 5 mM, pH 7·5) using a Teflon pestle (20 strokes). After centrifugation at 39 000*g* for 10 min at 4 °C, the pellets were resuspended in 'incubation buffer' (Tris-HCl 50 mM, $MgCl_2$ 10 mM, pH 7·5) and washed three times.

Platelet membranes were used immediately after

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their preparation; the membranes were incubated for 30 min at 25 °C in a shaking bath, in the presence of [^{3}H]rauwolscine (from New England Nuclear, Boston, MA; specific activity 75 or 87 Ci mmol^{-1}). For saturation experiments the concentration of [^{3}H]rauwolscine ranged from 0·1–10 nM, whereas inhibition experiments were performed using a fixed concentration of [^{3}H]rauwolscine (2·5 nM).

The final volume was 0·2 ml and the platelet membranes concentrations was 1 mg of protein ml^{-1} (Bradford 1976). At the end of the incubation, after the addition of 2 ml of cold buffer, bound and free ligand were separated by filtration through Whatman GF/C glass-fiber filters using a single port manifold system. Filters were rapidly washed with 20 ml of cold buffer (4 °C), dried and counted in a Triton-toluene mixture in a Packard β-counter with an efficiency of 49%.

Specific binding was defined as the [^{3}H]rauwolscine binding displaceable by 10^{-4} M yohimbine both in Scatchard and competition experiments. The equilibrium dissociation constant (K_D) and the maximal number of binding sites (B_{max}) were calculated from plots according to Scatchard (Scatchard 1949). Competition curves involving α_2-adrenoceptor agonists were analysed using a non linear least-squares fitting technique with statistical analysis, as recently published (Benfenati & Guardabasso 1984) and used in α- and β-receptor experiments (Vago et al 1983; Vago et al 1984). This technique provides estimate and standard errors for the affinity constants of the competing ligand and the values of K_D, K_I and IC50. These values are expressed as mean ± s.e. Drugs used were: guanabenz (L.P.B., Milan, Italy); (+)-, (−)-D 600 (gallopamil) (Grünenthal, Stolberg, Germany); St 600 (5-fluor-2-methyl-2-imidazolidinylidenbenzamin - monohydrochloride), B-HT 920 (6-allyl-2-amino-5,6,7,8-tetrahydro -4H- thiazolo-(4,5-*d*)-azepine dihydrochloride), B-HT 933 (2-amino-6-ethyl-4,5,7,8-tetrahydro-6*H*-oxazolo-(5,4-*d*)-azepine dihydrochloride), clonidine (Boehringer Ingelheim, Florence, Italy); guanfacine (Sandoz, Basel, Switzerland); nifedipine (Bayer AG, Wuppertal, Germany). All other drugs and compounds were obtained from commercial sources.

Results

Saturability, affinity and specificity of [^{3}H]rauwolscine binding sites. Fig. 1 shows the pattern of [^{3}H]rauwolscine binding in a particulate fraction derived from human platelets. Analysis of the data by Scatchard plot (an example in Fig. 1, inset) demonstrates a single class of binding sites with a K_D of 3·45 ± 0·6 nM. The analysis gives an estimate of 247 ± 15 fmol ligand bound (mg protein)$^{-1}$ (n = 15). The ability of various catecholamines to compete for binding sites on a particulated fraction from human platelets (Table 1) was characteristic of an α-receptor: indeed, the potency series for the (−)-stereoisomers tested was: (−)-adrenaline > (−)-noradrenaline > (−)-isoprenaline. Competition for the binding sites was stereospecific since the (+)-stereoisomers of noradrenaline and adrenaline were 10-fold less potent than were the (−)-stereoisomers (Table 1) (n = 4).

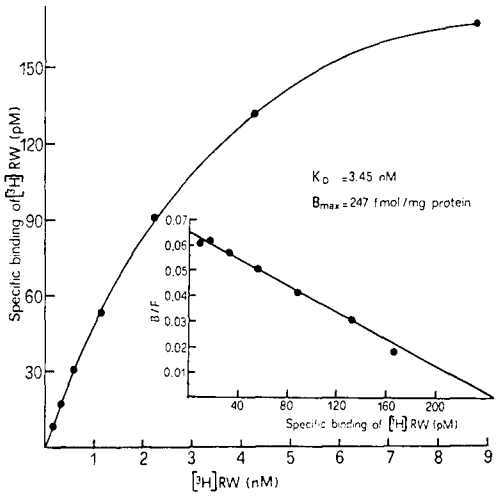

FIG. 1. Specific binding of [^{3}H]rauwolscine to human platelet membranes as function of increasing [^{3}H]rauwolscine concentrations. The inset shows the Scatchard plot demonstrating the values of K_D = 3·45 nM and of B_{max} = 247 fmol mg protein^{-1}.

Effects of various agonists on α_2-adrenoceptors. The inhibition of [^{3}H]rauwolscine binding by a variety of α_2-adrenoceptor agonists is shown in Fig. 2A (imidazoline-like drugs) (n = 4), Fig. 2B (azoloazepine derivatives) (n = 4) and in Fig. 2C (β-phenethylamines) (n = 4) and in Table 1.

Imidazoline-like drugs were the most potent as a group, with IC50 values ranging from 8·6 × 10^{-8} M (guanabenz) to 1·8 × 10^{-7} M (guanfacine). B-HT 920 competed for the binding sites at a concentration much lower than B-HT 933 (IC50 = 2·9 × 10^{-7} M and 2·8 × 10^{-5} M, respectively) (Fig. 2B and Table 1); among the β-phenethylamines (−)-adrenaline was the most active in competing for binding sites (Fig. 2C and Table 1).

Table 1. Values of IC50 of various adrenergic agonists in inhibiting binding of [^{3}H]rauwolscine in human platelet membranes.

	IC50 (M)
Guanabenz	8·6 ± 0·8 × 10^{-8}
Clonidine	1·6 ± 0·5 × 10^{-7}
Guanfacine	1·8 ± 0·6 × 10^{-7}
B-HT 920	2·9 ± 0·3 × 10^{-7}
B-HT 933	2·8 ± 1·2 × 10^{-5}
St 600	1·3 ± 0·3 × 10^{-7}
(−)-Adrenaline	3·4 ± 0·5 × 10^{-7}
(+)-Adrenaline	3·0 ± 0·5 × 10^{-6}
(−)-Noradrenaline	1·1 ± 0·2 × 10^{-6}
(+)-Noradrenaline	1·5 ± 1·0 × 10^{-5}
α-Methylnoradrenaline	2·3 ± 0·6 × 10^{-6}
(−)-Isoprenaline	5·5 ± 1·1 × 10^{-5}

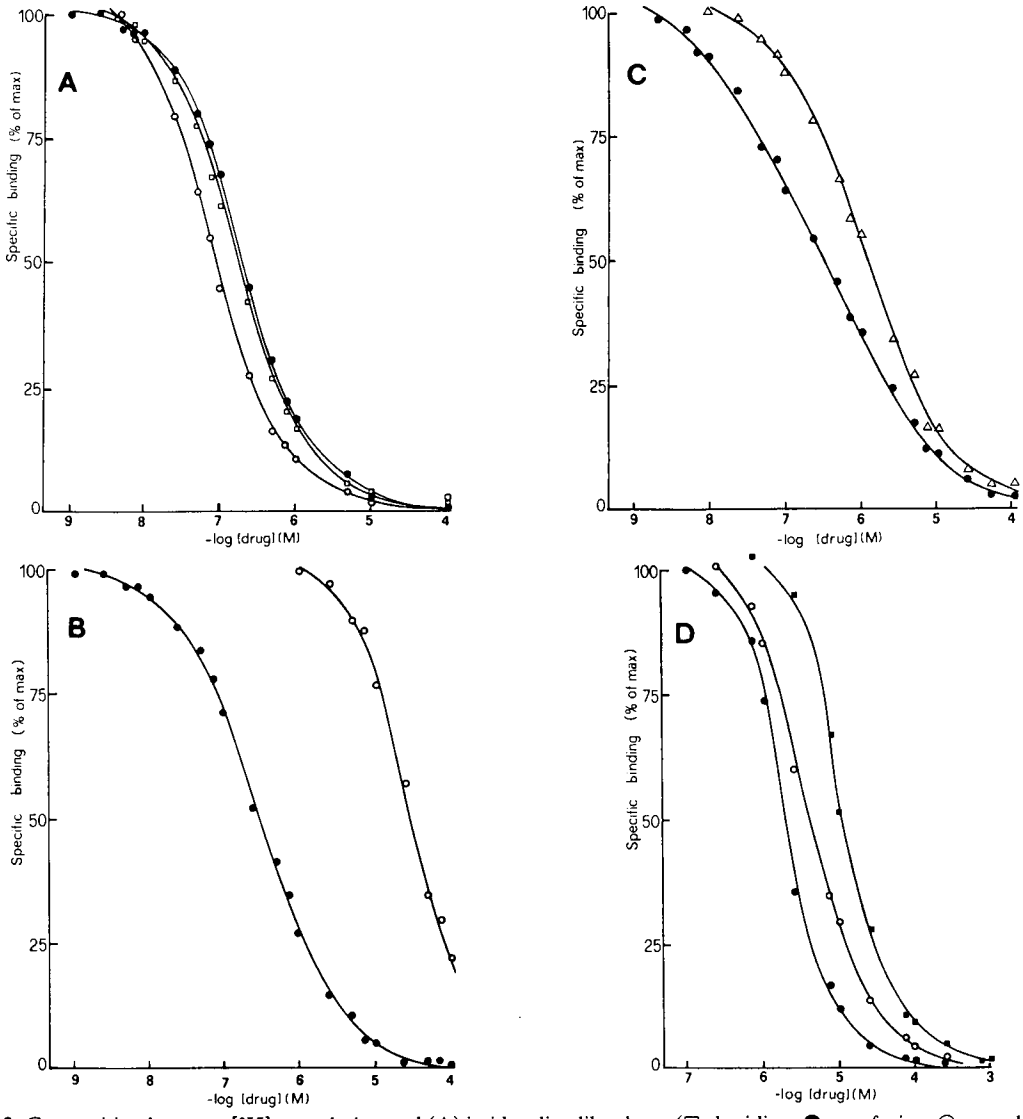

FIG. 2. Competition between [^{3}H]rauwolscine and (A) imidazoline like-drugs (□ clonidine, ● guanfacine, ○ guanabenz), (B) B-HT 920 (●) and B-HT 933 (○) (azoloazepine derivatives). (C) β-phenethylamines (● (−)-adrenaline, △ (−)-noradrenaline), (D) verapamil (○) and its methoxyderivatives (−)-D 600 (●) and (+)-D 600 ■) for binding to the α_2-adrenoceptors on human platelet membranes.

Interaction of calcium channel blockers with the α_2-adrenoceptors. The competition between [^{3}H]rauwolscine and various concentrations of verapamil and of its methoxyderivatives (±)-D 600, (+)-D 600 and (−)-D 600 for binding to the α_2-adrenoceptors on membrane preparated from human platelets is shown in Fig. 2D. Verapamil was the most potent with IC50 = 3·6 ± 0·9 × 10^{-6} (n = 2); the IC50 values of (−)-D 600 and (+)-D 600 were 1·6 ± 0·4 × 10^{-6} and 1·0 ± 1·0 × 10^{-5}, respectively (n = 4). Verapamil (Table 2) competed for [^{3}H]rauwolscine binding to platelet membrane receptors similarly in the presence and absence of GTP, indicating that it is an antagonist (data not shown) (n = 2) (Motulsky et al 1983). Diltiazem and nifedipine did not bear structural resemblance to verapamil, in fact, they did not compete for [^{3}H]rauwolscine binding even at high concentrations (Table 2) (n = 1 for each drug).

Discussion

Our experiments provide further evidence that human platelet membranes possess specific, saturable and high-affinity binding sites, with the characteristics of α_2-adrenoceptors. When we evaluated the potency of various α_2-adrenoceptor agonists on these receptors we

found that imidazoline-like drugs were more potent than B-HT 920 and β-phenethylamines in inhibiting [^{3}H]rauwolscine binding. This potency series, i.e. imidazoline-like drugs > B-HT 920 > β-phenethylamines, was similar to that observed in radioligand binding studies in rat brain (Timmermans & van Zwieten 1982) and in cardiovascular studies in the pithed rat (Timmermans & van Zwieten 1982), suggesting a similarity not only between platelet α_2-adrenoceptors and brain α_2-adrenoceptors but also between human platelet α_2-adrenoceptors and rat vascular smooth muscle adrenoceptors.

Table 2. Values of IC50 of various calcium blockers in inhibition binding of [^{3}H]rauwolscine in human platelet membranes.

	IC50 (M)
Verapamil	$3{\cdot}6 \pm 0{\cdot}9 \times 10^{-6}$
(−)-D 600	$1{\cdot}6 \pm 0{\cdot}4 \times 10^{-6}$
(+)-D 600	$1{\cdot}0 \pm 1{\cdot}0 \times 10^{-5}$
Diltiazem	$>10^{-4}$
Nifedipine	$>10^{-4}$

The lack of binding studies on the peripheral pre-synaptic α_2-adrenoceptors hinders a direct comparison of the potency profile on these receptors: however in animal models of pre-synaptic α_2-adrenoceptors such as the electrically-evoked twitch response of rat vas deferens and guinea-pig ileum (Mottram 1983), B-HT 920 was less potent than imidazoline-like drugs; the same was observed in the pre-synaptic cardiac α_2-adrenoceptors in the pithed rat (van Meel et al 1981). Furthermore, β-phenethylamines have been repeatedly shown to have a low affinity for pre-synaptic α_2-adrenoceptors (Mottram 1982). These data suggest a close analogy between peripheral pre-synaptic α_2-adrenoceptors and platelet α_2-adrenoceptors since both receptors respond to agonists with the same potency series, i.e. imidazoline-like drugs > B-HT 920 > β-phenethylamines.

Calcium entry blockers have been shown to modulate the vascular smooth muscle vasoconstrictor effects of α_2- and α_1-adrenoceptor agonists (van Zwieten et al 1983; Cavero et al 1983). Furthermore, verapamil enhances [^{3}H]noradrenaline efflux in rabbit hypothalamic slices (Galzin & Langer 1983), an effect related to the blockade of pre-synaptic α_2-adrenoceptors. Verapamil also antagonizes [^{3}H]rauwolscine binding in human platelets (Motulsky et al 1983). This antagonism is stereospecific, (−)-D 600 being more potent than (+)-D 600. Nifedipine and diltiazem, which have been shown to antagonize the pressor effect of post-synaptic α_2-adrenoceptor stimulation by B-HT 920 (van Zwieten et al 1983), in our study do not affect [^{3}H]rauwolscine binding.

These data suggest that while the calcium entry blocker verapamil antagonizes α_2-adrenoceptors in a stereospecific manner, the inhibitory action of nifedipine and diltiazem on vascular smooth muscle contractility is independent of inhibition of α_2-adrenoceptors.

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