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Further Studies on α_2-Adrenoceptor Subtypes Involved in the Modulation of [^{3}H]Noradrenaline and [^{3}H]5-Hydroxytryptamine Release from Rat Brain Cortex Synaptosomes

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Abstract—Three selective α_{2A}- or α_{2B}-adrenergic antagonists (BRL-44408, BRL-41992 and imiloxan) were used in the present study designed to classify the presynaptic α_2-auto- and heteroreceptors in the rat brain cortex. The rank order of potency in antagonizing the inhibitory effect of (−)-noradrenaline or clonidine on the K^+-induced [^{3}H]noradrenaline and [^{3}H]5-hydroxytryptamine (5-HT) release from superfused synaptosomes was BRL-44408 ≥ BRL-41992»imiloxan. The same rank order was found for the affinities of these compounds for [^{3}H]yohimbine binding in human platelet membranes, containing only α_{2A}-adrenoceptors, but does not correlate with the known affinities for α_{2B}-adrenoceptors (BRL-41992 ≥ imiloxan > BRL-44408). These data support the conclusion that presynaptic α_2-auto- and heteroreceptors in rat brain cortex do not belong to the α_{2B}-subtype and suggest that the modulation of noradrenaline and 5-HT release may be mediated by the α_{2A}-subtype.

The heterogeneity of α_2-adrenoceptors is now widely accepted. Bylund et al (1988) defined the α_{2A}- and the α_{2B}-adrenoceptors as having respectively low and high affinity for prazosin. In opossum-kidney derived cells (OK cells) an α_{2C}-subtype was described, whose characteristics are similar to the α_{2B}-subtype (Blaxall et al 1991). The α_2-adrenoceptors found in the bovine pineal gland have been termed α_{2D}, since their pharmacology is different from the other three (Simmoneaux et al 1991). Functional studies have shown that the α_2-adrenoceptors modulating noradrenaline release from guinea-pig isolated uretha (Alberts 1992), from guinea-pig submucosal plexus (Shen et al 1990), from rat vas deferens (Connaughton & Docherty 1990) and from rabbit brain cortical slices (Limberger et al 1991) possess the α_{2A} pharmacology while noradrenaline release in the rat atrium or in cat cerebral arteries appeared to be modulated by the α_{2B}-subtype (Connaughton & Docherty 1990; Arribas et al 1991). We have concentrated our studies on the α_2-adrenoceptors present in the rat brain cortex, where they modulate noradrenaline and 5-hydroxytryptamine (5-HT) release by auto- and heteroreceptors, respectively (Mulder et al 1978; Gothert et al 1981; Raiteri et al 1983). Comparing the findings from binding studies and from release studies in synaptosomes, we previously concluded that presynaptic α_2-auto- or heteroreceptors do not belong to the α_{2B}-(or α_{2C}) subtype but are similar to the α_{2A}-subtype (Gobbi et al 1990a), based on data obtained with a selective α_{2A}-agonist (oxymetazoline), a selective $\alpha_{2B/C}$-antagonist (prazosin) and two non-selective antagonists (idazoxan and 1-(2-pyrimidinyl)piperazine) (Gobbi et al 1990b). The aim of the present study was to verify this conclusion using other recently described compounds: imiloxan, a selective α_{2B}-antagonist (Michel et al 1990); BRL-44408, a selective α_{2A}-antagonist (Young et al 1989); BRL-41992, first described as a selective α_{2B}-antagonist by binding studies (Young et al 1989) and functional studies (Limberger et al 1991) even if more recent binding data casts doubt on this selectivity (Gleason & Hieble 1991).

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Materials and Methods

[^{3}H]Noradrenaline and [^{3}H]5-HT release

Male CRL:CD(SD)BR rats (Charles River, Italy), 150 g, were killed by decapitation and their cerebral cortices were rapidly dissected and homogenized in 40 vol ice-chilled 0·32 M sucrose, pH 7·4, in a glass homogenizer with a Teflon pestle. The homogenates were centrifuged at 1000 *g* for 5 min and the supernatants centrifuged again at 12 000 *g* for 20 min to yield the crude synaptosomal pellet (P_2) (Raiteri et al 1983). The synaptosomes were resuspended in about 20 vol Krebs-Henseleit buffer of the following composition (mM): NaCl 125, KCl 3, $CaCl_2$ 1·2, $MgSO_4$ 1·2, NaH_2PO_4 1, $NaHCO_3$ 22, glucose 10, aerated with 95% O_2–5% CO_2, pH 7·2–7·4. The suspension was then added to an equal volume of the same buffer containing [^{3}H]5-HT or [^{3}H]noradrenaline (12·5 and 45 Ci mmol^{-1}, respectively; Amersham, Buckinghamshire, UK) at a final concentration of 0·06 μM. After 15 min incubation at 37°C, the solution was diluted with fresh buffer and 5-mL samples (about 10–15 mg initial tissue) were distributed onto 0·65 μm cellulose nitrate filters (Perdomini, Verona, Italy) in a 16-chamber superfusion apparatus maintained at 37°C. The synaptosomes were layered onto the the filters by aspiration from below under moderate vacuum.

Superfusion was started (t = 0 min) at a rate of 0·5 mL min^{-1} with standard medium. From t = 47 to t = 50 min, synaptosomes were depolarized by replacing the medium with one containing a higher KCl concentration (15 mM, replacing an equimolar concentration of NaCl) with or without the agonist ((−)-noradrenaline, 0·3 and 1 μM, or clonidine 0·3 μM). Antagonists (BRL-44408, BRL-41992 and imiloxan) were present in the superfusion medium from

t = 40 min. Fractions (2 min each) were collected from t = 42 until t = 60 min and were counted for radioactivity, as were the filters, in 8 mL Atom-Light (New England Nuclear, Boston, MA, USA).

The percentage fractional release rate (FRR) was calculated as 100 times the amount of radioactivity released into each 2-min fraction divided by the total radioactivity present on the filter at the start of that fraction. The K^+-evoked overflow, representing the difference between the FRR in the presence and absence of 15 mM K^+, was calculated by subtracting the mean FRR before (t = 44–48) and after (t = 56–60) the stimulus from each FRR obtained during the stimulus (t = 48–56) and summing these differences; the overflow is therefore expressed as the percentage per 8 min (note that the effect of K^+, added at t = 47, was detectable only 1 min later, since the fluid takes about 1·5 min to flow from the filters to the collecting vials). Under these conditions the radioactivity released by high K^+ consists mainly of unmetabolized [^{3}H]noradrenaline and [^{3}H]5-HT (Maura et al 1985; Gobbi et al 1992).

[^{3}H]Yohimbine binding in human platelets

[^{3}H]Yohimbine binding in human platelets was measured as described by Cheung et al (1982). Blood (50 mL) was collected from male volunteers with 3% sodium citrate as anticoagulant. Platelet-rich plasma (PRP) was obtained by centrifuging the blood at 270 *g* at 10°C for 15 min. PRP was then centrifuged at 27000 *g* for 10 min at 4°C and the resulting pellet resuspended in ice-cold lysing buffer (5 mM Tris-HCl plus 5 mM EDTA) and left for 1–2 min before being homogenized by 10 strokes of a motor-driven glass-Teflon homogenizer. The suspension was then centrifuged at 27 000 *g* at 4°C for 10 min. The pellet was washed once with lysing buffer by gentle resuspension and then centrifuged at 27 000 *g* at 4°C for 10 min. It was finally washed by resuspension in ice-cold assay buffer (50 mM Tris-HCl containing 0·5 mM EDTA, 0·1% ascorbic acid, pH 7·5), followed by centrifugation at 27000 *g* at 4°C for 10 min. The final pellet was resuspended in 13 mL of assay buffer and 250 μL portions were incubated at 22°C for 30 min with [^{3}H]yohimbine (Amersham, 84 Ci $mmol^{-1}$, final concentration 2 nM) and different concentrations of competing drugs. Phentolamine 10 μM was used to determine nonspecific binding. The samples were rapidly filtered under vacuum on Whatman GF/B filters, which were washed three times with 3 mL buffer and counted for radioactivity in 8 mL Filter Count (Packard, Groningen, The Netherlands).

[^{3}H]Yohimbine binding in rabbit brain cortex

Brain cortex from New Zealand Rabbits (HY/CR) was homogenized in Tris-HCl (50 mM containing 5 mM EDTA, pH 7·4) using an Ultra Turrax TP-1810. The homogenate was centrifuged at 50 000 *g* for 10 min and resuspended three times in the ice-cold buffer. The membranes were incubated for 30 min at 25°C with 2 nM [^{3}H]yohimbine and different concentrations of competing drugs. Phentolamine (10 μM) was used to determine nonspecific binding. The samples were rapidly filtered under vacuum on Whatman GF/C filters, which were washed three times with 3 mL buffer and counted for radioactivity.

Drugs

Sources of drugs were as follows: (−)-noradrenaline HCl from Fluka (Buchs, Switzerland); clonidine from Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT, USA); oxymetazoline HCl from Bracco (Milano, Italy); prazosin HCl from Pfizer (Groton, CT, USA); BRL-44408 and BRL-41992 from SmithKline Beecham (Great Burgh, Surrey, UK); imiloxan from Syntex (Palo Alto, CA, USA).

Compounds were dissolved in distilled water, except for BRL-44408 and BRL-41992 which were dissolved in dimethylsulphoxide (DMSO). The final concentration of DMSO, 0·1–1% in the superfusion buffer of the release experiments, had no effect; in the binding experiments, the final concentration of DMSO was 1%, which slightly reduced specific [^{3}H]yohimbine binding.

Results and Discussion

The basal outflow of tritium from cortical synaptosomes preloaded with [^{3}H]noradrenaline and superfused for 47 min was constant, with an FRR of 1·8 ± 0·2%/2 min, corresponding to 3·2 ± 0·4 nCi/2 min (mean ± s.e.m. of 7 experiments). Under these conditions the K^+-induced [^{3}H]noradrenaline overflow was 12·0 ± 1·1%/8 min, corresponding to 19·3 ± 1·9 nCi/8 min (mean ± s.e.m. of 7 experiments).

In the [^{3}H]5-HT release experiments, basal tritium outflow was stable with an FRR of 2·7 ± 0·7%/2 min, corresponding to 0·7 ± 0·1 nCi/2 min (mean ± s.e.m. of 7 experiments). The K^+-induced [^{3}H]-5HT overflow was 11·4 ± 1·1%/8 min, corresponding to 2·4 ± 0·5 nCi/8 min (mean ± s.e.m. of 7 experiments).

The antagonists used in the present study (BRL-44408, BRL-41992 and imiloxan up to 1 μM) had no effect on basal outflow or K^+-induced overflow of [^{3}H]noradrenaline and [^{3}H]5-HT (data not shown).

Both BRL-44408 and BRL-41992 dose-dependently antagonized the inhibitory effect of (−)-noradrenaline, with similar IC50 values (39 and 42 nM on [^{3}H]noradrenaline release, respectively; 22 and 50 nM on [^{3}H]5-HT release, respectively). Imiloxan, up to 1 μM, had no effect (Fig. 1). Similar results were obtained using 0·3 μM clonidine as agonist (Table 1); in this case complete antagonism was found with 1 μM BRL-44408 and BRL-41992, whereas the same concentration of imiloxan had no effect. The three compounds were also tested on [^{3}H]yohimbine binding in human platelets to check for their affinity for the α_{2A}-adrenoceptors; there was no difference between the two compounds, BRL-44408 being only three times more active than BRL-41992, with imiloxan only one-twentieth to one-fiftieth as active (Table 2). Table 2 also shows that BRL-44408 and BRL-41992 had similar affinity for [^{3}H]yohimbine binding sites in the rabbit brain cortex; this area appears to contain only α_{2A}-adrenoceptors since the IC50 values of oxymetazoline and prazosin were respectively 3·3 nM and 6·7 μM, with slopes not different from one.

Release data showed that both BRL-44408 and BRL-41992, at nM concentrations, antagonized the inhibitory effect of (−)-noradrenaline at the α_2-auto- or heteroreceptors in rat cortical synaptosomes; imiloxan, however, was not active up to 1 μM. Similarly, 1 μM of BRL-44408 or BRL-41992 completely antagonized the inhibitory effect of cloni-

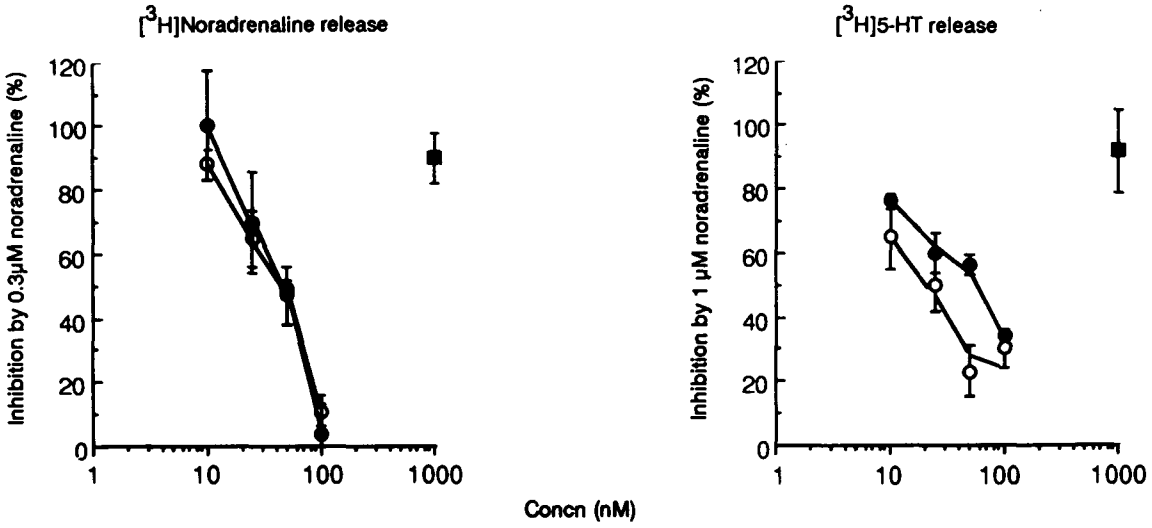

FIG. 1. Concentration-effect curves of BRL-44408 (○), BRL-41992 (●) and imiloxan (■) in antagonizing the inhibitory effect of (−)-noradrenaline on K^+-induced [^{3}H]noradrenaline or [^{3}H]5-HT release. Two experiments, each in duplicate, were performed for each antagonist. For each experiment, the inhibition obtained with (−)-noradrenaline, in the absence of the antagonist, was fixed at 100. The values in the graphs are therefore the mean ± s.e.m. of the percentages obtained with the different concentrations of antagonists. The absolute inhibition by 0·3 or 1 μM (−)-noradrenaline on the K^+-induced [^{3}H]noradrenaline or [^{3}H]5-HT release was 33·2 ± 6·8 and 31·0 ± 3·6%, respectively (means ± s.e.m., n = 4 experiments).

Table 1. Antagonism by 1 μM imiloxan, BRL-44408 or BRL-41992 on the inhibitory effect of 0·3 μM clonidine on K^+-induced [^{3}H]noradrenaline or [^{3}H]5-HT release.

Drug	[^{3}H]Noradrenaline release (% control)	[^{3}H]5-HT release (% control)
Clonidine	76 ± 5*	81 ± 2*
Clonidine + imiloxan	81 ± 2*	89 ± 1†*
Clonidine + BRL-44408	112 ± 4††	102 ± 4††
Clonidine + BRL-41992	99 ± 5††	98 ± 3††

*$P < 0.01$ compared with control, †$P < 0.05$, ††$P < 0.01$ compared with clonidine alone. Each value is the mean ± s.e.m. of 9–12 replicates from 4 different experiments.

dine while 1 μM imiloxan had no such effect; these last experiments were designed to replicate, with our model, the data by Limberger et al (1991) (see below). In agreement with these data, BRL-44408 and BRL-41992 had similar potencies ($pA_2 = 11$ and 17 nM, respectively, against UK-14304) at α_2-autoreceptors, as measured in electrically stimulated rat brain cortex slices (Trendelenburg et al personal communication).

The rank order of potency at the auto- or heteroreceptors (BRL-44408 = BRL-41992 > imiloxan) is therefore parallel to the rank order of affinity for the α_{2A}-adrenoceptors (BRL-44408 ≥ BRL-41992 > imiloxan). These data are in agreement with a previous report (Gobbi et al 1990a) showing that prazosin, an α_{2B}-antagonist behaving similarly to imiloxan in binding experiments (Michel et al 1990), was not active on auto- and heteroreceptors while oxymetazoline, an α_{2A}-antagonist, had high potency; moreover, the potency of idazoxan and pyrimidinylpiperazine in release experiments was proportional to their affinity for α_{2A}-adrenoceptors. These and the present data support the conclusion that presynaptic α_2-auto- or heteroreceptors in rat cortex do not belong to the α_{2B}- (or α_{2C} (Blaxall et al 1991)) subtype and suggest their similarity with the α_{2A}-subtype. A similar conclusion was suggested by Maura et al (1992) describing the different pharmacology of auto- and heteroreceptors.

Table 2. Affinities (IC50 values, nM) of BRL-44408, BRL-41992 and imiloxan for α_{2A}-adrenoceptors in human platelets and rabbit brain cortex incubated with [^{3}H]yohimbine.

	Human platelets		Rabbit brain cortex	
	Exp 1	Exp 2	Exp 1	Exp 2
BRL-44408	7·9 ± 0·6 (0·9)	4·5 ± 1·9 (1·3)	8·4 ± 0·6 (0·9)	6·4 ± 0·5 (0·9)
BRL-41992	25·7 ± 1·9 (0·9)	14·9 ± 6·6 (1·0)	32·3 ± 2·2 (0·9)	21·5 ± 1·8 (0·9)
Imiloxan	443 ± 57 (1·1)	472 ± 48 (1·2)	—	—
Prazosin	3404 ± 263 (0·9)	15271 ± 6865 (1·0)	6826 ± 1568 (1·1)	6685 ± 1360 (1·0)
Oxymetazoline	9·9 ± 0·7 (0·9)	1·6 ± 0·2 (1·2)	3·8 ± 0·2 (1·0)	2·8 ± 0·4 (1·0)

At least eight drug concentrations, each in duplicate, were tested on the binding of [^{3}H]yohimbine 2 nM. The IC50 values ± s.e.m. and the slopes (in parentheses) were calculated by nonlinear fitting (using Allfit program, DeLean et al (1978)) of the inhibition curves. The results from two different experiments are shown.

The involvement of α_{2D}-subtypes cannot at present be ruled out, even if these receptors are partially sensitive to prazosin (Simmoneaux et al 1991).

Limberger et al (1991), studying the release of [^{3}H]noradrenaline induced by electrical stimulation in rabbit brain cortical slices, found that the inhibitory effect of 0·3 μM clonidine was completely antagonized by 1 μM BRL-44408. However, at variance with our data, 1 μM of either BRL-41992 or prazosin was not active. Complete concentration-response curves gave estimated pA_2 values of 7·8 for BRL-44408 and only 6·2 for BRL-41992. These and other data led the authors to suggest that 'the presynaptic α_2-autoreceptors in rabbit brain cortex are markedly different from the α_{2B}-subtype and probably belong to the prazosin-insensitive α_{2A}-subtype'. However, the lack of any marked difference in affinities of the two BRL compounds for the α_{2A}-adrenoceptors, also indicated by the binding data by Gleason & Hieble (1991), does not support that conclusion; if affinity for the α_{2A}-subtype is the critical point for potency at the autoreceptor, then BRL-41992 should be much more potent than described by Limberger et al (1991). These results could suggest that the rabbit brain cortex contains subtypes of α_{2A}-adrenoceptors, recognized with different affinity by the two BRL compounds. Consequently, we may speculate that their similar IC50 values for [^{3}H]yohimbine binding sites in the rabbit brain cortex is due to the fact that the α_{2A}-autoreceptors represent only a small fraction of the total α_{2A}-receptor. These results confirm the differences already described between α_2-adrenoceptors in rat and rabbit brain cortex (Limberger et al 1989). However, the difficulties in using the current classification to interpret all the data raise some questions about the real meaning of this classification obtained by binding studies in selected tissues from various animal species and its general application to other preparations.

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