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Pharmacological characterization of presynaptic α_2-autoreceptors in rat submaxillary gland and heart atrium

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1 The pharmacological properties of presynaptic α_2-autoreceptors were studied in rat isolated submaxillary glands and atria. Tissue pieces were preincubated with [^{3}H]-noradrenaline, then superfused with medium containing desipramine, and stimulated electrically. In one series of experiments, pEC_{30} values of 12 α-adrenoceptor antagonists were determined, i.e., negative logarithms of concentrations that increased the electrically evoked overflow of tritium by 30%. In another series, pK_D values of 9 α-adrenoceptor antagonists against the release-inhibiting effect of 5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline (UK 14304), and of 3 antagonists against the release-inhibiting effect of methoxamine, were determined.

2 In submaxillary glands, the pEC_{30} values of the antagonists correlated well with their pK_D values against UK 14304 ($r = 0.93$). The same was true for atria ($r = 0.92$).

3 In submaxillary glands, the pK_D values of 3 antagonists against UK14304 were very similar to their pK_D values against methoxamine, with a maximal difference of 0.4. The same was true for atria where the maximal difference was 0.3.

4 The pEC_{30} values obtained in submaxillary glands correlated significantly with those obtained in atria ($r = 0.81$). The same was true for the pK_D values ($r = 0.79$). However, the pEC_{30} and pK_D values also indicated consistent differences between the two tissues.

5 It is concluded that the sites of action of the imidazoline UK 14304 (α_2-selective), the phenylethylamine noradrenaline, and the phenylethylamine methoxamine (α_1-selective) are exclusively α_2-adrenoceptors. There is no indication for presynaptic α_1-adrenoceptors or for an effect of UK 14304 mediated by presynaptic imidazoline receptors. The α_2-autoreceptor population in the submaxillary gland differs from that in the atrium.

6 Comparison with studies from the literature indicates that the submaxillary autoreceptors are closely similar to the α_{2D} radioligand binding site found in the bovine pineal gland and probably the rat submaxillary gland. The atrial autoreceptors also conform best to this site, but the agreement is more limited; the atrial autoreceptors may represent a type related to, but distinct from, the α_{2D} site, or a mixture of different types.

Keywords: α_1-Adrenoceptor; α_2-adrenoceptor; imidazoline receptor; presynaptic α_2-autoreceptor; receptor classification; rat heart atrium; rat submaxillary gland; sympathetic nervous system; noradrenaline release

Introduction

Studies on presynaptic α-autoreceptors led to the view that α-adrenoceptors are not homogeneous (Starke, 1972) but comprise at least two classes, α_1 and α_2 (Langer, 1974; for review see Docherty, 1989; Ruffolo *et al.*, 1991; Wilson *et al.*, 1991). Studies on presynaptic α-autoreceptors also first indicated heterogeneity within the α_2 class (Doxey & Everitt, 1977; Dubocovich, 1979). A species difference between the rabbit and the rat, for example, was a consistent finding (Reichenbacher *et al.*, 1982; Ennis, 1985; Lattimer & Rhodes, 1985; Alabaster *et al.*, 1986; Limberger *et al.*, 1989).

More systematic attempts to subclassify α_2-adrenoceptors were based on radioligand binding techniques (Cheung *et al.*, 1982; Bylund, 1985). They have led to the differentiation of four subclasses: α_{2A}, occurring for example on human platelets and HT29 human colonic carcinoma cells and characterized, *inter alia*, by low affinity for prazosin (Bylund *et al.*, 1988); α_{2B}, occurring for example in neonatal rat lung, with relatively high affinity for prazosin (Bylund *et al.*, 1988); α_{2C}, occurring for example in an opossum kidney (OK) cell line, also with relatively high affinity for prazosin (Blaxall *et al.*, 1991); and α_{2D}, occurring in the bovine pineal gland (Simonneaux *et al.*, 1991) and perhaps some rat tissues (Michel *et al.*, 1989; Paris *et al.*, 1990), with low prazosin affinity.

To which of these subgroups, if any, do the presynaptic α_2-autoreceptors belong? From data in the literature it was suggested that the autoreceptors were generally α_{2B} (Bylund *et al.*, 1988), that they might be α_{2C} in rat cerebral cortex and submaxillary gland (Blaxall *et al.*, 1991), or that some might be α_{2D} (Simonneaux *et al.*, 1991). Four studies, to our knowledge, have addressed the question directly, measuring the release of [^{3}H]-noradrenaline or postsynaptic responses to neural stimulation. In contrast to the suggestions mentioned, the autoreceptors were classified as α_{2A} in rat vas deferens (Connaughton & Docherty, 1990), rat brain cortex (Gobbi *et al.*, 1990), rabbit brain cortex (Limberger *et al.*, 1991) and guinea-pig urethra (Alberts, 1992); only in rat atrium the autoreceptors seemed to be α_{2B} (Connaughton & Docherty, 1990). However, the classification of the rat atrial receptors as α_{2B}, i.e. prazosin-sensitive, must be viewed with caution since the sympathetic nerves of this tissue have been postulated to possess (highly prazosin-sensitive) α_1-adrenoceptors (Kobinger & Pichler, 1980; Docherty, 1984).

We describe here a functional characterization of α_2-autoreceptors in rat submaxillary glands and atria. Rat atrial autoreceptors were chosen in order to re-examine their α_{2B} classification. Rat submaxillary gland autoreceptors had been important for the concept that α_2-autoreceptors were generally α_{2B} (Bylund, 1988; Bylund *et al.*, 1988). After our experiments were finished, Smith *et al.* (1992) published a comparison of α_2-autoreceptors in rat vas deferens, atrium and submaxillary gland in which the α_{2B} character of the

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atrial receptors was confirmed and the submaxillary receptors, like those of the vas deferens, were classified as α_{2A}. Our study suggests a reinterpretation of some findings of Smith *et al.* (1992).

Methods

Experiments with submaxillary glands and atria were carried out in parallel. Male Wistar rats weighing 160 to 240 g were decapitated. Submaxillary glands and atria were dissected free and cut into pieces of 4–6 mg. Six to eight pieces were preincubated with [^{3}H]-noradrenaline 0.1 μmol l^{-1} in 2 ml medium (37°C for 30 min) and subsequently washed five times with 3 ml of [^{3}H]-noradrenaline-free medium. One piece was transferred to each of twelve 0.16 ml superfusion chambers, six for glandular and six for atrial tissue. The tissue piece was held by a polypropylene mesh between platinum wire electrodes 6 mm apart. Tissues were superfused for 210 min at 1 ml min^{-1}. There were five periods of electrical stimulation (rectangular pulses of 2 ms width and 15 V cm^{-1} voltage drop, yielding a current strength of 24 mA). The first stimulation period was given after 40 min of superfusion (180 pulses, 3 Hz); it was not used for determination of tritium overflow. The following periods were applied after 70 (S_1), 110 (S_2), 150 (S_3) and 190 min (S_4) of superfusion. The collection of successive 5 min superfusate samples began after 60 min of superfusion. At the end, the tissue was solubilized in 0.5 ml Soluene-350 (Packard, Frankfurt am Main, Germany).

The preincubation and superfusion media contained (mmol l^{-1}): NaCl 118, KCl 4.8, $CaCl_2$ 0.2, 2 or 2.5 (see below), $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, ascorbic acid 0.57, disodium EDTA 0.03 and corticosterone 0.01. The superfusion but not the preincubation medium also contained desipramine 1 μmol l^{-1}.

There were two kinds of experiment. In the first series, the effect of α-adrenoceptor antagonists on the electrically evoked overflow of tritium was determined. The concentration of $CaCl_2$ was 2 mmol l^{-1}. Each of the stimulation periods S_1 to S_4 consisted of 60 pulses/1 Hz. The antagonist under study was added at increasing concentrations from 20 min before to 20 min after the onset of S_2, S_3 and S_4 to yield cumulative antagonist concentration-response curves (see Figure 1, upper panels). In the second series of experiments, antagonist effects against the agonists 5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline (UK 14304) or methoxamine were determined. The concentration of $CaCl_2$ was 0.2 mmol l^{-1} for preincubation and 2.5 mmol l^{-1} for superfusion; the basal outflow of tritium was more stable under these conditions than at 2 mmol l^{-1} $CaCl_2$. Each of the stimulation periods S_1 to S_4 consisted of 10 trains of 6 pulses/100 Hz; the trains were delivered at intervals of 30 s; the total duration of a stimulation period, hence, was 4.5 min. The antagonist under study was present throughout superfusion, and either UK 14304 or methoxamine was added at increasing concentrations from 20 min before to 20 min after the onset of S_2, S_3 and S_4 to yield cumulative agonist concentration-response curves. One common set of concentration-response data for UK 14304, given alone, served for comparison with UK 14304 curves obtained in presence of each of the antagonists; the same was done for methoxamine. Concentration-response curves for rauwolscine were also determined under the conditions of the second series (Figure 1, lower panels).

The outflow of tritium was expressed as fractional rate (min^{-1}). The stimulation-evoked overflow was calculated by subtraction of the basal outflow and was expressed as a percentage of the tritium content of the tissue (Limberger *et al.*, 1989; 1991). For further evaluation of basal tritium efflux, ratios were calculated of the fractional rate during the 5 min before S_2, S_3 and S_4, and the fractional rate during the 5 min before S_1 (b_2/b_1, b_3/b_1, b_4/b_1). Ratios were also calculated for the electrically evoked overflow (S_2/S_1, S_3/S_1, $S_4/$

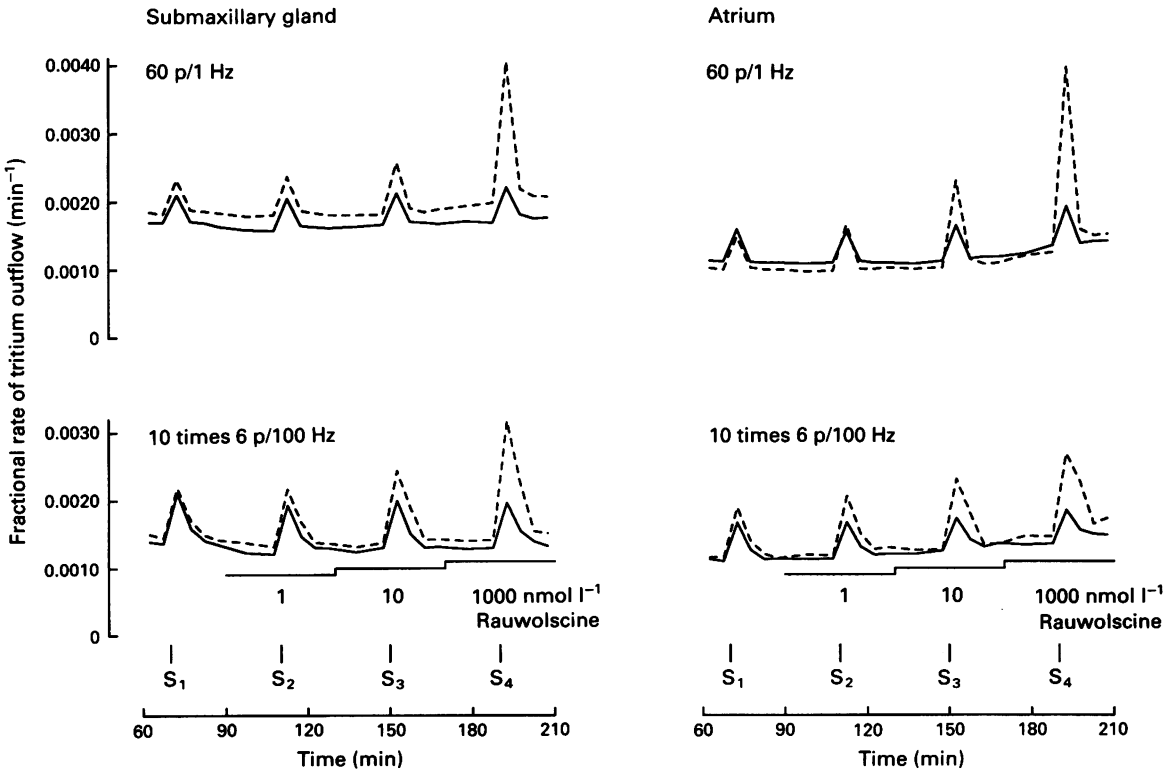

Figure 1 Time course of tritium outflow from rat submaxillary gland (left-hand panels) and atrium (right-hand panels), and effect of rauwolscine. The tissue was preincubated with [^{3}H]-noradrenaline and then superfused with medium containing desipramine 1 μmol l^{-1} and corticosterone 10 μmol l^{-1}. Each stimulation period (S_1–S_4) consisted either of 60 pulses/1 Hz (upper panels), or of 10 trains of 6 pulses/100 Hz, train interval 30 s (lower panels). Solvent (solid lines) or increasing concentrations of rauwolscine (dashed lines) were added as indicated. Abscissa scale, min of superfusion. Ordinate scale, mean fractional rate of tritium efflux. Each curve is the mean from two tissue fragments.

S_1). Moreover, the percentage change caused by an agonist or antagonist in each single piece of tissue was calculated, taking, as the reference value, the average corresponding S_2/S_1, S_3/S_1 and S_4/S_1 ratio obtained in experiments in which no drug was added before S_2, S_3 and S_4.

Antagonist pEC_{30} values (negative logarithms of concentrations that enhanced the evoked overflow of tritium by 30%) were interpolated from the averaged concentration-reponse curves. The effect of an antagonist against UK 14304 and methoxamine was quantitated as follows. A sigmoid curve was fitted to the weighted mean percentage inhibition values for the agonist given alone, and a second curve was simultaneously fitted to the weighted mean percentage inhibition values for the agonist in the presence of the antagonist, assuming a common maximal agonist effect and slope (equation No. 25 of Waud, 1976); this yielded the EC_{50} (concentration producing 50% of the maximal effect) of the agonist alone, and of the agonist in the presence of antagonist. The pair of EC_{50} values was taken to calculate the pK_D of the antagonist (Starke *et al.*, 1975), using the Gaussian law of error propagation. The common set of concentration-response data for 'agonist given alone' (see above) yielded different fitted curves, depending on which antagonist entered into the same fitting calculation. However, the differences were minimal. Concentration-response curves, EC_{50} values and maximal effects for 'agonist given alone' in Results are taken from the calculation in which rauwolscine 0.1 μmol l^{-1} was the antagonist.

Results are expressed as arithmetic means ± s.e.mean. Group means were compared with the Mann-Whitney test if Kruskal-Wallis analysis indicated a significant difference. In the case of multiple comparisons, the significance levels to be exceeded were adjusted according to Bonferroni. *n* is the number of tissue pieces.

Drugs

Purchased drugs were (-)-[ring-2,5,6-^{3}H]-noradrenaline, specific activity 56.7 Ci $mmol^{-1}$ (Du Pont, Dreieich, Germany), (±)-2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4-benzodioxane HCl (WB 4101) (Biotrend, Köln, Germany), corynanthine HCl, rauwolscine HCl and yohimbine HCl (Roth, Karlsruhe, Germany) and corticosterone (Sigma, Deisenhofen, Germany). The following drugs were kindly provided by the producers: (±)-methoxamine HCl (Burroughs Wellcome, London, U.K.); desipramine HCl and phentolamine methanesulphonate (Ciba-Geigy, Basel, Switzerland); prazosin HCl and 5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline tartrate (UK 14304) (Pfizer, Karlsruhe, Germany); (±)-idazoxan HCl (Reckitt & Colman, Kingston-upon-Hull, U.K.); (±)-imiloxan HCl (RS 21361; Syntex, Edinburgh, U.K.); (±)-2-[2H-(1-methyl-1,3-dihydroisoindole)methyl]-4,5-dihydroimidazole (BRL 44408) and (-)-1,2-dimethyl-2,3,9,13b-tetrahydro-1H-dibenzo[c,f]imidazo[1,5-a]azepine (BRL 41992) (SmithKline Beecham, Great Burgh, Epsom, Surrey, U.K.); 6-chloro-9-[(3-methyl-2-butenyl)oxy]-3-methyl-1H-2,3,4,5-tetrahydro-3-benzazepine maleate (SKF 104078) (Smith Kline and French, King of Prussia, Philadelphia, U.S.A.); 2-{2-[4-(o-methoxyphenyl) piperazin-1-yl] ethyl}-4,4-dimethyl-1,3 (2H,4H)-isoquinolinedione dihydrochloride (ARC 239) (Thomae, Biberach an der Riss, Germany). Drugs were dissolved in distilled water except WB 4101 (HCl 1 mmol l^{-1}), corticosterone (1,2-propanediol), BRL 44408 and BRL 41992 (HCl 10 mmol l^{-1}).

Results

Release-enhancing effect of antagonists

The first series of experiments examined the potency of 12 α-adrenoceptor antagonists at enhancing the release of [^{3}H]-noradrenaline. The slices were stimulated by trains of 60 pulses/1 Hz, conditions under which marked autoinhibition developed. The overflow of tritium evoked by S_1 amounted to 0.233 ± 0.004% of the tritium content of the tissue for the submaxillary gland ($n = 161$) and to 0.234 ± 0.006% for the atrium ($n = 154$). In control experiments without α-adrenoceptor antagonists, the basal outflow of tritium, when expressed as fractional rate, was approximately constant (glands) or increased slightly (atria), and the evoked overflow was similar from S_1 to S_4 (Figure 1, upper panels).

All antagonists tested increased the evoked overflow of tritium. Representative experiments with rauwolscine are shown in the upper panels of Figure 1, and concentration-response curves for rauwolscine, prazosin, SKF 104078, imiloxan and corynanthine in Figure 2. No attempt was made to obtain maximal effects, firstly because concentration-response curves of α-adrenoceptor antagonists on the release of noradrenaline often are bell-shaped, with non-specific inhibition coming into play at high concentrations (Borowski *et al.*, 1977), and secondly because high concentrations often accelerated the basal efflux of tritium (see below). The highest increases observed were by about 400%. The percentage increases caused by a given antagonist concentration often differed between submaxillary gland and atrium, some antagonists being more effective in the atrium, others in the submaxillary gland.

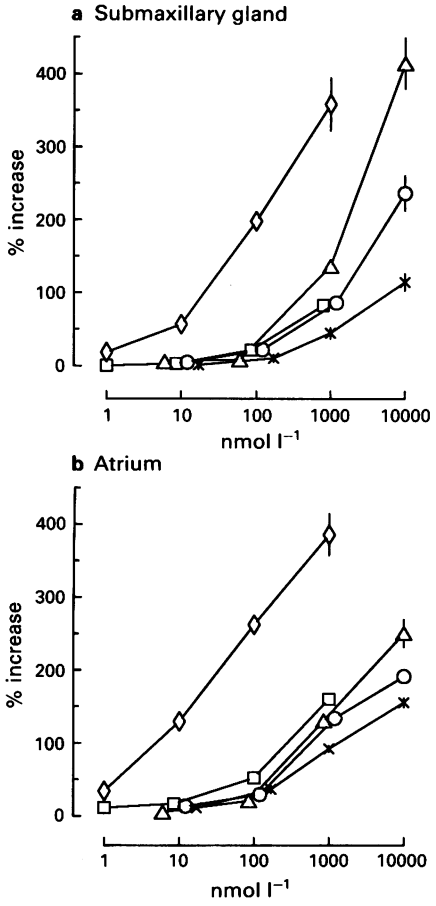

Figure 2 Effects of α-adrenoceptor antagonists on electrically evoked tritium overflow from rat submaxillary gland (a) and atrium (b). The tissue was preincubated with [^{3}H]-noradrenaline and then superfused with medium containing desipramine 1 μmol l^{-1} and corticosterone 10 μmol l^{-1}. Each stimulation period (S_1–S_4) consisted of 60 pulses/1 Hz. Rauwolscine (◇), prazosin (□), SKF 104078 (○), imiloxan (△) or corynanthine (×) was added at increasing concentrations before S_2, S_3 and S_4. Abscissae, antagonist concentration. Ordinates, percentage increase caused by antagonists, calculated from S_n/S_1 values. Means ± s.e.mean (vertical bars) of at least 4 tissues.

pEC_{30} values are summarized in Table 1, and the correlation of submaxillary with atrial pEC_{30} values is shown in Figure 3a. Imiloxan had similar pEC_{30} values in the two tissues, and the same was true for SKF 104078 and idazoxan. For other antagonists, however, the two pEC_{30} values differed by up to 0.8, with higher affinity in the submaxillary gland in some cases (points above line of identity in Figure 3a) and higher affinity in the atrium in others (points below line of identity), so that the correlation coefficient was only 0.81.

The basal outflow of tritium was not changed by most antagonists. Exceptions were increases caused by SKF 104078 10 μmol l^{-1} (by about 210%), prazosin 0.1 (by about 30%) and 1 μmol l^{-1} (by about 160%) and ARC 239 1 μmol l^{-1} (by about 30%). pEC_{30} values were calculated despite these increases, because the electrically evoked overflow remained well set off from the raised baseline; moreover, the acceleration of basal tritium efflux was small at concentrations close to the calculated EC_{30}.

Effect of antagonists against UK 14304 and methoxamine

The second series of experiments examined the potency of 9 α-adrenoceptor antagonists in attenuating the release-inhibiting effect of UK 14304, and the potency of 3 antagonists in attenuating the effect of methoxamine, under conditions of minimal autoinhibition. Autoinhibitory tone can be avoided when single electric pulses or brief high-frequency pulse trains are used (see Singer, 1988). Single pulses, however, and single brief high-frequency trains (for example 6 pulses/100 Hz) elicited too small an increase in tritium outflow for reliable measurement (data not shown). The conditions finally chosen (each stimulation period consisting of 10 trains of 6 pulses/100 Hz, train interval 30 s) were a compromise. On the one hand, the stimulation-evoked overflow peaks were sufficiently high, and on the other hand, autoinhibition was relatively minor as shown by a relatively small effect of rauwolscine (compare upper and lower panels of Figure 1); rauwolscine 1 μmol l^{-1}, for example, increased the evoked overflow of tritium by only 137.8 ± 6.6% in the submaxillary gland ($n = 6$) and 105.8 ± 11.5% in the atrium ($n = 6$) instead of the much greater increase observed with 60 pulses/1 Hz (by about 400%; Figure 2).

The overflow of tritium elicited by S_1, in the absence of

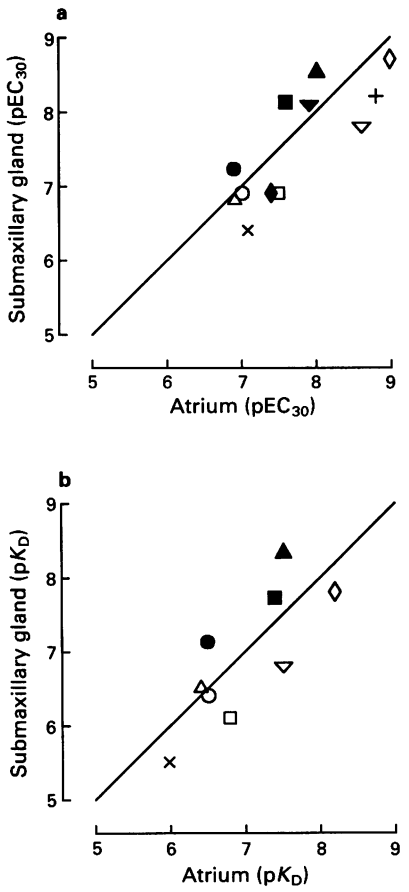

Figure 3 Correlation between antagonist affinity estimates for α_2-autoreceptors in rat atrium and submaxillary gland: (a) shows correlation between pEC_{30} values (negative logarithms of concentrations that increased evoked tritium overflow by 30%); (b) shows correlation between pK_D values (calculated from antagonism against UK 14304). Values are from Table 1. Lines are the lines of identity, not regression lines. Correlation coefficients are 0.81 (a) and 0.79 (b). Symbols: (▲) phentolamine; (◇) rauwolscine; (■) BRL 44408; (●) BRL 41992; (▽) WB 4101; (△) imiloxan; (○) SKF 104078; (□) prazosin; (×) corynanthine; (+) yohimbine; (▼) idazoxan; (◆) ARC 239.

Table 1 Potencies of α-adrenoceptor antagonists in increasing the evoked overflow of tritium (pEC_{30}) and in antagonizing the inhibitory effects of UK 14304 and methoxamine (pK_D)

α-Adrenoceptor antagonist	*Submaxillary gland* pEC_{30}	pK_D *(against UK 14304)*	pK_D *(against methoxamine)*	*Atrium* pEC_{30}	pK_D *(against UK 14304)*	pK_D *(against methoxamine)*
Phentolamine	8.5	8.3 ± 0.1	–	8.0	7.5 ± 0.1	–
Rauwolscine	8.7	7.8 ± 0.1	7.8 ± 0.6	9.0	8.2 ± 0.1	7.9 ± 0.3
BRL 44408	8.1	7.7 ± 0.1	–	7.6	7.4 ± 0.2	–
BRL 41992	7.2	7.1 ± 0.1	–	6.9	6.5 ± 0.2	–
WB 4101	7.8	6.8 ± 0.2	–	8.6	7.5 ± 0.2	–
Imiloxan	6.8	6.5 ± 0.1	6.7 ± 0.7	6.9	6.4 ± 0.2	6.5 ± 0.3
SKF 104078	6.9	6.4 ± 0.2	–	7.0	6.5 ± 0.2	–
Prazosin	6.9	6.1 ± 0.2	6.5 ± 0.3	7.5	6.8 ± 0.2	6.7 ± 0.2
Corynanthine	6.4	5.5 ± 0.2	–	7.1	6.0 ± 0.2	–
Yohimbine	8.2	–	–	8.8	–	–
Idazoxan	8.1	–	–	7.9	–	–
ARC 239	6.9	–	–	7.4	–	–

pEC_{30} values are negative logarithms of concentrations that increased evoked tritium overflow by 30%. pK_D values were calculated from antagonism against UK 14304 and methoxamine. Antagonist concentrations used to determine pEC_{30} were those of Figure 2 and 1–1000 nmol l^{-1} for other antagonists. Antagonist concentrations used to determine pK_D were those of Table 2; prazosin 0.1 μmol l^{-1} was also tested against UK 14304; prazosin 0.1 μmol l^{-1} increased basal tritium outflow only slightly and did not increase electrically evoked overflow; the pK_D against UK 14304 was 6.3 ± 0.3 in submaxillary gland and 6.7 ± 0.4 in atrium. Means ± s.e.mean. Each pEC_{30} is based on 8–16 and each pK_D on 10–12 tissues, controls and tissues that received agonist only not included.

antagonists, was higher in this series of experiments (Table 2) than in the first one (see above) despite the equal number of pulses (60). Possible reasons are the weaker autoinhibition and the slightly higher concentration of Ca^{2+} in this (2.5 mmol l^{-1}) than in the first series (2 mmol l^{-1}). In control experiments (no antagonist, no agonist), the basal outflow of tritium again remained approximately constant (submaxillary glands) or increased slightly (atria), and the evoked overflow was similar from S_1 to S_4 (Figure 1, lower panels). All antagonists, when present throughout superfusion, increased S_1 values (Table 2). As in controls, S_1 to S_4 overflow values were similar when an antagonist was present throughout superfusion, without subsequent addition of an agonist (data not shown). Prazosin 1 μmol l^{-1} markedly accelerated the basal efflux of tritium (Table 2).

UK 14304 and methoxamine, given alone, decreased the evoked overflow of tritium (experiments represented by circles in Figures 4 and 5). An asymptotic maximum of inhibition was reached in the case of UK 14304 (Figure 4). Sigmoidal curve fitting yielded EC_{50} values of UK 14304 of 6.2 ± 1.1 nmol l^{-1} (submaxillary gland) and 6.8 ± 1.2 nmol l^{-1} (atrium), and maximal inhibition values of 91.7 ± 2.2% (submaxillary gland) and 82.6 ± 3.4% (atrium) (from 14 gland and 11 atrial pieces). UK 14304 did not change the basal efflux of tritium. In contrast, no obvious asymptotic maximum of inhibition was reached for methoxamine (Figure 5). Concentrations higher than methoxamine 100 μmol l^{-1} were not used because, whereas methoxamine 100 μmol l^{-1} only slightly accelerated the basal efflux of tritium, higher concentrations caused a drastic increase. Despite the lack of a clear maximum, the curve fitting calculation converged to give EC_{50} values of methoxamine of 20.9 ± 16.2 μmol l^{-1} (submaxillary gland) and 8.0 ± 3.8 μmol l^{-1} (atrium), and maximal inhibition values of 76.0 ± 15.3% (submaxillary gland) and 75.4 ± 11.1% (atrium) (from 13 gland and 11 atrial pieces).

The antagonism of rauwolscine, imiloxan and prazosin against UK 14304 and methoxamine is shown in Figures 4 and 5, respectively–rauwolscine, imiloxan and prazosin were the three drugs tested against either agonist. pK_D values are summarized in Table 1, and the correlation between submaxillary gland and atrium is shown in Figure 3b. Three comparisons are of particular interest: between pEC_{30} and pK_D values within one tissue; between pK_D values measured against UK 14304 and those measured against methoxamine; and between submaxillary glands and atria.

A comparison of pK_D with pEC_{30} values in the same tissue

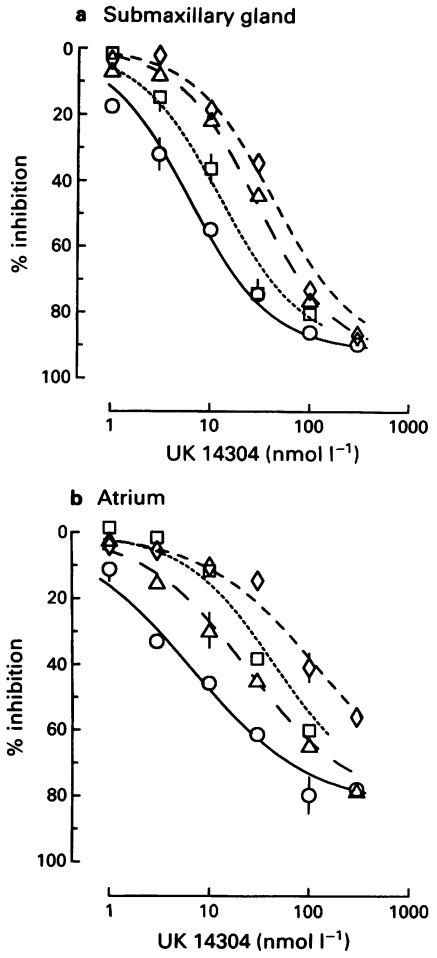

Figure 4 Effect of UK 14304 on electrically evoked tritium overflow from rat submaxillary gland (a) and atrium (b), and interaction with α-adrenoceptor antagonists. The tissue was preincubated with [^{3}H]-noradrenaline and then superfused with medium containing either desipramine 1 μmol l^{-1} and corticosterone 10 μmol l^{-1} only (○) or, in addition, rauwolscine 0.1 μmol l^{-1} (◇), imiloxan 1 μmol l^{-1} (△) or prazosin 1 μmol l^{-1} (□). Each stimulation period (S_1–S_4) consisted of 10 trains of 6 pulses/100 Hz, train interval 30 s. UK 14304 was added at increasing concentrations before S_2, S_3 and S_4. Abscissae, concentration of UK 14304. Ordinates, percentage inhibition caused by UK 14304, calculated from S_n/S_1 values. Means ± s.e.mean (vertical lines) of at least 6 tissues.

Table 2 Basal outflow (b_1) and evoked overflow of tritium (S_1) from rat submaxillary gland and atrium

α-Adrenoceptor antagonist present throughout superfusion (μmol l^{-1})		Submaxillary gland			Atrium		
		b_1 (min^{-1})	S_1 (% of tissue tritium)	n	b_1 (min^{-1})	S_1 (% of tissue tritium)	n
–		0.00161 ± 0.00003	0.439 ± 0.016	40	0.00103 ± 0.00003	0.434 ± 0.021	36
Phentolamine	0.3	0.00154 ± 0.00004	0.721 ± 0.038**	18	0.00096 ± 0.00002	0.761 ± 0.043**	22
Rauwolscine	0.1	0.00153 ± 0.00005	0.811 ± 0.031**	33	0.00100 ± 0.00002	0.704 ± 0.036**	39
BRL 44408	1	0.00159 ± 0.00008	0.650 ± 0.037**	18	0.00085 ± 0.00003**	0.707 ± 0.036**	18
BRL 41992	1	0.00142 ± 0.00003**	0.747 ± 0.040**	18	0.00102 ± 0.00003	0.584 ± 0.023**	18
WB 4101	0.3	0.00181 ± 0.00010	0.747 ± 0.046**	18	0.00103 ± 0.00002	0.807 ± 0.038**	18
Imiloxan	1	0.00148 ± 0.00004	0.767 ± 0.032**	33	0.00096 ± 0.00003	0.639 ± 0.026**	32
SKF 104078	1	0.00172 ± 0.00007	0.654 ± 0.037**	18	0.00107 ± 0.00003	0.533 ± 0.023*	18
Prazosin	1	0.00343 ± 0.00006**	0.650 ± 0.030**	30	0.00317 ± 0.00004**	0.552 ± 0.025*	36
Corynanthine	3	0.00154 ± 0.00005	0.684 ± 0.035**	18	0.00118 ± 0.00004	0.640 ± 0.022**	18

The tissue was preincubated with [^{3}H]-noradrenaline and then superfused with medium containing desipramine 1 μmol l^{-1}, corticosterone 10 μmol l^{-1} and the α-adrenoceptor antagonists indicated. Electrical stimulation (S_1) consisted of 10 trains of 6 pulses/100 Hz, train interval 30 s. b_1 represents the outflow of tritium in the collection period immediately before S_1, i.e. from 65–70 min of superfusion, expressed as fractional rate (min^{-1}); the absolute outflow averaged 2.52 ± 0.13 nCi/5 min (submaxillary gland; n = 40) and 1.65 ± 0.09 nCi/5 min (atrium; n = 36) in controls. S_1 represents the overflow of tritium elicited by the period of electrical stimulation applied after 70 min of superfusion, expressed as a percentage of tissue tritium; the absolute overflow averaged 1.40 ± 0.08 nCi (submaxillary gland) and 1.45 ± 0.12 nCi (atrium) in controls. Means ± s.e.mean from n tissues. Significant differences from control (no α-adrenoceptor antagonist): *P < 0.05; **P < 0.01.

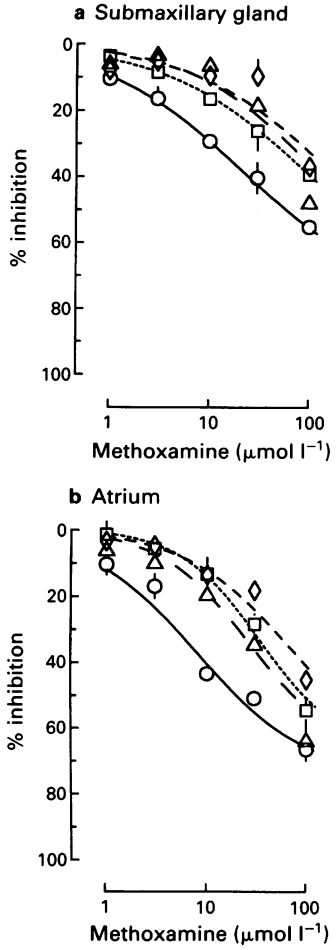

Figure 5 Effect of methoxamine on electrically evoked tritium overflow from rat submaxillary gland (a) and atrium (b), and interaction with α-adrenoceptor antagonists. The tissue was preincubated with [^{3}H]-noradrenaline and then superfused with medium containing either desipramine 1 μmol l^{-1} and corticosterone 10 μmol l^{-1} only (○) or, in addition, rauwolscine 0.1 μmol l^{-1} (◇), imiloxan 1 μmol l^{-1} (△) or prazosin 1 μmol l^{-1} (□). Each stimulation period (S_1–S_4) consisted of 10 trains of 6 pulses/100 Hz, train interval 30 s. Methoxamine was added at increasing concentrations before S_2, S_3 and S_4. Abscissae, concentration of methoxamine. Ordinates, percentage inhibition caused by methoxamine, calculated from S_n/S_1 values. Means ± s.e.mean (vertical lines) of at least 5 tissues.

shows that pK_D values were consistently lower, the difference varying between 0.1 and 1.1 (Table1). Because of the systematic character of this deviation, there was good correlation between pEC_{30} values and the pK_D values against UK 14304. The regression equation was, for the submaxillary gland: $pEC_{30} = 0.85 \times pK_D + 1.63$ ($r = 0.93$; $n = 9$ antagonists; $P < 0.001$); for the atrium: $pEC_{30} = 0.99 \times pK_D + 0.68$ ($r = 0.92$; $n = 9$ antagonists; $P < 0.001$).

In either tissue, the pK_D values of rauwolscine, imiloxan and prazosin against UK 14304 on the one hand and against methoxamine on the other hand were closely similar, with a maximal difference of 0.4 (Table 1). The pK_D values against methoxamine varied more than those against UK 14304 (s.e.mean in Table 1), presumably because the inhibition was smaller and no clear maximum was reached in the case of methoxamine (Figure 5).

The third comparison, between the two tissues, indicates that imiloxan had similar pK_D values in submaxillary gland and atrium, and the same was true for SKF 104078. However, for other antagonists the two pK_D values differed by up to 0.8, with higher affinity in the gland in some cases (points above line of identity in Figure 3b) and higher affinity in the atrium in others (points below line of identity), so that the correlation coefficient was only 0.79. Parts (a) and (b) of Figure 3 show that a given antagonist occupies approximately the same position with respect to the line of identity, irrespective of whether pEC_{30} values (Figure 3a) or pK_D values (Figure 3b) are correlated.

Discussion

The electrically evoked overflow of tritium in the present kind of experiment reflects neuronal release of [^{3}H]-noradrenaline. In accord with this view, tetrodotoxin 0.3 μmol l^{-1} abolished the evoked overflow (not shown). Two procedures were used to characterize the presynaptic α-adrenoceptors known to modulate the release of noradrenaline in rat submaxillary glands (Filinger *et al.*, 1978) and atria (Majewski *et al.*, 1981). The first method assessed the overflow-increasing potency of antagonists under conditions of pronounced α_2-autoinhibition. Since desipramine was present, the increases were not due to blockade of the reuptake of released [^{3}H]-noradrenaline but to facilitation of its neuronal release, and the pEC_{30} values of the antagonists were in fact estimates of their autoreceptor affinities. The second procedure assessed antagonist potencies against exogenous agonists and yielded pK_D values. An autoinhibition-free release of noradrenaline is optimal for such determinations (see Starke, 1987). We did not obtain an autoinhibition-free and yet reliably measurable release in the present study; however, with the protocol chosen the distortion by autoinhibition (examples in Limberger *et al.*, 1989) was minimized.

The pEC_{30} values correlated well with the pK_D values (against UK 14304) both in the submaxillary gland ($r = 0.93$) and the atrium ($r = 0.92$; $P < 0.001$). The close correlation indicates an inner consistency of the data: two independent estimations of antagonist affinity led to similar results. The pEC_{30} values were all higher than the pK_D values, presumably because less than 50% receptor occupation sufficed for a 30% increase in [^{3}H]-noradrenaline release. pEC_{30} values were also determined in rat submaxillary glands and atria by Connaughton & Docherty (1990) and Smith *et al.* (1992). Of 12 pEC_{30} values that can be compared, 10 were higher (by 0.3–1.1) in our experiments than in those of these authors; the remaining 2 were similar. Differences in methods may explain the tendency for greater antagonist effects in the present study; for instance, we used much smaller pieces of tissue, a longer exposure to antagonists and a lower frequency of stimulation.

No evidence for imidazoline receptors and α_1-adrenoceptors

The good correlation of the pK_D (against UK 14304) and pEC_{30} values (antagonism against released noradrenaline) is noteworthy for a second reason. It shows that the sites of action of UK 14304, an imidazoline, and of endogenous noradrenaline, a phenylethylamine, had the same pharmacological properties, those of an α-adrenoceptor. (The same holds true for the phenyethylamine methoxamine; see below.) Presynaptic imidazoline receptors distinct from the α-autoreceptors have been demonstrated in rabbit aorta and pulmonary artery (Göthert & Molderings, 1991; Molderings *et al.*, 1991). No indication for an effect of UK 14304 on imidazoline receptors was found in rat submaxillary gland and atrium (see also Starke, 1987; Docherty, 1989).

Rat atria were postulated to possess release-inhibiting α_1- in addition to α_2-autoreceptors. The suggestion was based on *in vivo* experiments in which noradrenaline release was estimated indirectly as an increase in heart rate (Kobinger & Pichler, 1980; Docherty, 1984). However, *in vitro* findings seemed to support the suggestion: in rat isolated atria, the α_1-selective agonist (Starke *et al.*, 1975) methoxamine

(10 μmol l^{-1}) reduced the release of [^{3}H]-noradrenaline, an effect antagonized by prazosin (0.1 μmol l^{-1}), in apparent agreement with the occurrence of presynaptic α_1-adrenoceptors (Story *et al.*, 1985). The present experiments confirm the observation of Story *et al.* (1985), but the more quantitative approach questions their conclusion. First, the pK_D value of prazosin against methoxamine (6.7; Table 1, atrium) is far too low for an α_1-adrenoceptor (range 8–11.2 in Figure 1 of Wilson *et al.*, 1991). Second, the α_2-selective antagonists, rauwolscine (Weitzell *et al.*, 1979) and imiloxan (RS 21361; Michel & Whiting, 1981; Lattimer & Rhodes, 1985), also antagonized methoxamine and their pK_D values (rauwolscine 7.9, imiloxan 6.5) are far too high for an α_1-adrenoceptor (rauwolscine range 5–7, imiloxan range 3.1–4.3 in Figure 1 of Wilson *et al.*, 1991). Third, each of the three antagonists was equipotent against methoxamine and the α_2-selective agonist (Cambridge, 1981) UK 14304 (Table 1, Figures 4 and 5), indicating that the two agonists acted at the same site. Finally, the common site was an α_2-adrenoceptor: the pK_D values of prazosin, rauwolscine and imiloxan demonstrate this (Figure 1 of Wilson *et al.*, 1991), as do other findings such as the high potencies of yohimbine and idazoxan at increasing the release of [^{3}H]-noradrenaline. α_2-Adrenoceptor agonist effects of methoxamine are known for other tissues (e.g., Guimaraes *et al.*, 1987). Generally speaking, the existence of presynaptic α_1-adrenoceptors at postganglionic sympathetic axons remains doubtful (Starke, 1987; Docherty, 1989; Shinozuka *et al.*,1991).

Properties of the α_2-autoreceptors

To which of the subgroups defined by radioligand binding do the α_2-autoreceptors belong? The comparison will be based on the pK_D values determined against UK 14304 (Table 1), but a comparison based on the pEC_{30} values leads to the same conclusions. The low absolute affinity of the autoreceptors for prazosin (pK_D against UK 14304 6.1 in submaxillary gland and 6.8 in atrium) already argues against α_{2B} properties (binding pK_D 7.6–8.3 in Table 2 of Simonneaux *et al.*, 1991). Affinity correlations as well as affinity ratios (see below) indicate that the properties of the submaxillary autoreceptors agree well with those of the α_{2D} binding site in bovine pineal gland as defined by Simonneaux *et al.* (1991). The atrial autoreceptors also resemble the α_{2D} site most; here, however, the similarity is much more limited; moreover, consistent differences between atrial and submaxillary autoreceptors indicate that they are not the same.

Correlations are summarized in Table 3. The submaxillary autoreceptor affinities correlate significantly with binding affinities to the bovine pineal gland α_{2D} site ($r = 0.90$) but not with α_{2A}, α_{2B} and α_{2C} binding affinities. Moreover, the regression coefficient is close to unity in the case of the α_{2D} correlation. For the atrial autoreceptors, the answer is doubtful. Although correlation is best with the bovine pineal α_{2D} site ($r = 0.95$), the correlation with the neonatal rat lung α_{2B} site is almost as good, and there is also significant correlation with α_{2A} and α_{2C} sites.

Ratios of antagonist K_D values, calculated from the present experiments and from binding data, are summarized in Table 4. Several ratios clearly differentiate the rat submaxillary autoreceptor from α_{2A}, α_{2B} and α_{2C}. For example, the phentolamine/rauwolscine ratio is 8.6 at α_{2A} sites (the two values of Table 4 averaged) but 0.32 at the submaxillary autoreceptor, and the prazosin/WB 4101 ratio is 240 at α_{2A} sites (on average) but 5 at the autoreceptor. On the other hand, 5 of 6 submaxillary autoreceptor ratios are relatively close to the α_{2D} ratios in bovine pineal gland (less than 5 fold difference; exception: WB 4101/phentolamine). As to atrial autoreceptors, several ratios also set them apart from α_{2A}, α_{2B} and α_{2C}. For example, the BRL 44408/BRL 41992 ratio is 131 at the neonatal rat lung α_{2B} site but 0.13 at atrial autoreceptors, a 1008 fold difference, and the BRL 44408/prazosin ratio is 49.8 at the α_{2B} site but 0.25 at the autoreceptors, a 199 fold difference. The atrial autoreceptors agree better, but less than the submaxillary autoreceptors, with α_{2D}: 3 of 6 ratios are relatively close to the α_{2D} ratios in bovine pineal gland (less than 5 fold difference), and for the remaining 3 ratios the greatest difference is 8 fold (corynanthine/phentolamine).

It has been suggested that binding sites for radiolabelled α_2-adrenoceptor antagonists in rat submaxillary gland (Michel *et al.*, 1989) and rat jejunum epithelial cells (Paris *et al.*, 1990) also represent α_{2D}-adrenoceptors (Paris *et al.*, 1990; Lanier *et al.*, 1991; Simonneaux *et al.*, 1991). Seven antagonists could be used to compare our functional autoreceptor affinities with affinities for the [^{3}H]-rauwolscine binding site in rat submaxillary gland (Michel *et al.*, 1989). The correlation between the submaxillary autoreceptors and the submaxillary [^{3}H]-rauwolscine binding site is excellent, correlation coefficient 0.98 (Table 3; see also Figure 6a). The correlation between the atrial autoreceptors and the submaxillary [^{3}H]-rauwolscine binding site, in contrast, is relatively poor, correlation coefficient 0.78 (Table 3; see also Figure 6b). Moreover, all antagonist K_D ratios for submaxillary autoreceptors are very close to those for the submaxillary [^{3}H]-rauwolscine binding site (Table 4). In the case of the atrial autoreceptors, there are differences, the largest one between a phentolamine/rauwolscine ratio of 5 for atrial autoreceptors and of 0.18 for the submaxillary binding site (Table 4). If the submaxillary binding site is α_{2D}, this com-

Table 3 Correlation between antagonist affinity estimates for α_2-autoreceptors and various α_2 binding sites

Tissue used for [^{3}H]-antagonist binding	*Submaxillary gland autoreceptors*		*Atrial autoreceptors*		*Number of antagonists*
	Correlation coefficient	*Regression coefficient*	*Correlation coefficient*	*Regression coefficient*	
Human platelets (α_{2A})[a]	0.81	0.88	0.85	1.29	5
HT29 cell line (α_{2A})[b]	0.73	0.79	0.87*	1.23	6
Neonatal rat lung (α_{2B})[b]	0.74	0.60	0.93**	1.00	6
OK cell line (α_{2C})[b]	0.50	0.58	0.85*	1.31	6
Bovine pineal gland (α_{2D})[c]	0.90*	0.92	0.95**	1.28	6
Rat submaxillary gland[d]	0.98**	0.92	0.78*	0.91	7

Autoreceptor affinity estimates are pK_D values calculated from antagonism against UK 14304 (from Table 1). Binding site affinity estimates are pK_D values calculated from inhibition of the binding of [^{3}H]-yohimbine or [^{3}H]-rauwolscine (from references quoted). Shown are correlation coefficients and slopes of the regression of binding pK_D on autoreceptor pK_D. Antagonists were phentolamine, rauwolscine, WB4101, SKF 104078, prazosin and corynanthine except for correlations with human platelet binding sites (no binding data on SKF 104078) and rat submaxillary gland binding sites (imiloxan in addition; the correlations with submaxillary binding sites are shown in Figure 6). Significant differences from 0: $*P<0.05$; $**P<0.01$.

[a] Cheung *et al.* (1982).
[b] Blaxall *et al.* (1991).
[c] Simonneaux *et al.* (1991).
[d] Michel *et al.* (1989).

Table 4 Ratios of K_D values of α-adrenoceptor antagonists

Tissue	*Phentolamine/ rauwolscine*	*Corynanthine/ phentolamine*	*Prazosin/ corynanthine*	*Prazosin/ WB 4101*	*Prazosin/ phentolamine*	*WB 4101/ phentolamine*	*BRL 44408/ BRL 41992*	*BRL 44408/ prazosin*
				Autoreceptors				
Rat submaxillary gland	0.32	631	0.25	5.0	158	31.6	0.25	0.03
Rat atrium	5.0	31.6	0.16	5.0	5.0	1.0	0.13	0.25
				Binding sites				
Human platelets (α_{2A})[a,b]	3.6	47.9	4.0	216	190	0.88	0.02	0.003
HT29 cell line (α_{2A})[c,d]	13.6	22.0	2.4	263	52.7	0.20	0.61	0.007
Neonatal rat lung (α_{2B})[b,c]	8.0	27.8	0.05	0.62	1.5	2.4	131	49.8
OK cell line (α_{2C})[c]	226	2.9	0.54	55.6	1.5	0.03	–	–
Bovine pineal gland (α_{2D})[e]	0.91	248	0.14	14.0	34.2	2.5	–	–
Rat submaxillary gland[f]	0.18	457	0.30	7.6	135	17.8	–	–

Shown are ratios for the α_2-autoreceptors of rat submaxillary gland and atrium (K_D calculated from antagonism against UK 14304; from Table 1) and for various α_2 binding sites (K_D calculated from inhibition of the binding of [^{3}H]-yohimbine or [^{3}H]-rauwolscine; from references quoted).
[a] Cheung *et al.* (1982).
[b] Young *et al.* (1989) (BRL 44408/BRL 41992 and BRL 44408/prazosin).
[c] Blaxall *et al.* (1991).
[d] Gleason & Hieble (1991) (BRL 44408/BRL 41992 and BRL 44408/prazosin).
[e] Simonneaux *et al.* (1991).
[f] Michel *et al.* (1989).

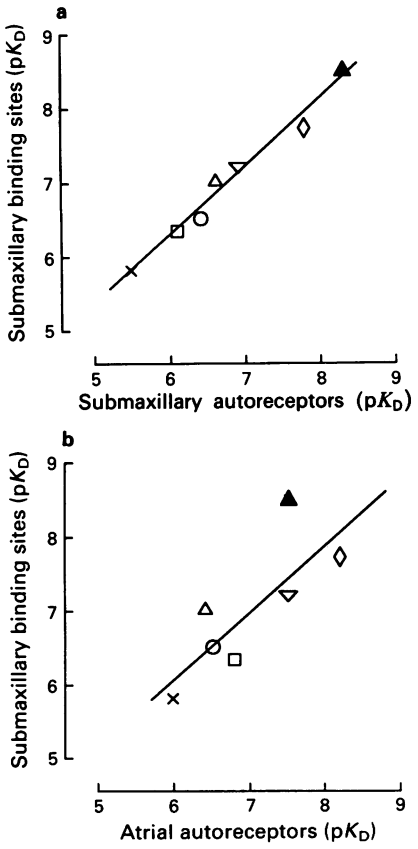

Figure 6 Correlation between antagonist affinity estimates for α_2-autoreceptors and the [^{3}H]-rauwolscine binding site in rat submaxillary gland: (a) shows correlation for rat submaxillary gland autoreceptors and (b) for rat atrial autoreceptors. Autoreceptor affinity estimates are pK_D values calculated from antagonism against UK 14304 (from Table 1). Binding site affinity estimates are pK_D values calculated from inhibition of the binding of [^{3}H]-rauwolscine (from Michel *et al.*, 1989). Symbols as in Figure 3. Regression line in (a): binding $pK_D = 0.92 \times$ autoreceptor $pK_D + 0.79$; in (b): binding $pK_D = 0.91 \times$ autoreceptor $pK_D + 0.61$.

parison confirms both the α_{2D} character of the submaxillary autoreceptors and the deviation in the case of the atrial autoreceptors.

Previous α_2-autoreceptor characterizations

Smith *et al.* (1992) have recently classified the α_2-autoreceptors of rat submaxillary gland and rat vas deferens as α_{2A} and those of rat atrium as α_{2B}. They based their conclusion on a comparison with their own binding data for α_{2A} and α_{2B} sites and did not attempt a classification in terms of α_{2C} or α_{2D}. We confirm the difference between the atrial and the submaxillary autoreceptors. The difference is real: the same similarities and differences were detected in two independent assessments of autoreceptor properties (Figure 3a versus Figure 3b). On the other hand, we would like to suggest that the results of Smith *et al.* (1992) are open to re-interpretation as far as subtype assignments are concerned. In the case of the vas deferens autoreceptor, a comparison of the pK_D (pA_2) values of Docherty's group (Connaughton & Docherty, 1989; Smith *et al.*, 1992) with the binding sites listed in our Table 3 yields the best correlation with the bovine pineal α_{2D} site ($r = 0.87$) and the submaxillary [^{3}H]-rauwolscine (α_{2D}?) binding site ($r = 0.98$). Moreover, the vas deferens autoreceptor pK_D values correlate very well with our submaxillary autoreceptor (α_{2D}) values ($r = 0.98$). These correlations suggest that the vas deferens autoreceptor is α_{2D} rather than α_{2A}; some K_D ratios point in the same direction. There are too few antagonists to compare the submaxillary autoreceptor pEC_{30} values of Smith *et al.* (1992) with literature data. However, none of their findings excludes α_{2D}, and the excellent agreement between the submaxillary and the vas deferens autoreceptors found by Smith *et al.* (1992) makes a re-interpretation as α_{2D} possible. Finally, the findings of Connaughton & Docherty (1989) and Smith *et al.* (1992) on atrial autoreceptors are not incompatible with a closer similarity to α_{2D} than α_{2B}. For example, their atrial autoreceptor pEC_{30} values correlate better with pK_D values for the α_{2D} site in bovine pineal gland ($r = 0.90$) than with pK_D values for the α_{2B} site in neonatal rat lung ($r = 0.71$) (binding studies

listed in our Table 3; see already Connaughton & Docherty, 1989).

Differences between the presynaptic autoreceptors of rat and rabbit tissues early indicated a non-homogeneity of the α_2 class (see Introduction). The difference may be due to the occurrence of predominantly α_{2A}-autoreceptors in rabbits (Limberger *et al.*, 1991) and α_{2D}-autoreceptors in rats.

In conclusion, the imidazoline adrenoceptor agonist UK 14304 inhibits the release of noradrenaline in rat submaxillary glands and atria exclusively by an effect on α_2-autoceptors and not on imidazoline receptors. The selective α_1-adrenoceptor agonist methoxamine also causes inhibition only through α_2-adrenoceptors; no evidence for presynaptic α_1-adrenoceptors was found. The pharmacological properties of the atrial α_2-autoreceptors, as assessed by two independent methods, differ consistently from those of the submaxillary α_2-autoreceptors. The properties of the submaxillary autoreceptors agree so well with those of α_{2D} binding sites that the two, apart from species differences, may be identical. The atrial autoreceptors also resemble α_{2D} binding sites most. They may belong to a subtype similar to, but not identical with, α_{2D}. However, the existence of a mixed α_2-autoreceptor population in rat atria cannot be ruled out.

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