The α_2 -adrenoceptors of the human retinoblastoma cell line (Y79) may represent an additional example of the α_{2C} -adrenoceptor

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1 In agreement with the literature, correlation of the ability of a series of agonists and antagonists to displace [³H]-rauwolscine binding shows the α_2 -adrenoceptors of HT29 cells, NG108-15 cells, OK cells and homogenates of rat sublingual gland to represent four distinct subtypes.

2 [³H]-rauwolscine also bound with high affinity ($K_D = 0.30 \pm 0.10$ nM) to a human retinoblastoma cell line (Y79). Specific binding represents 73% of total binding, and a B_{max} of 38 ± 1 fmol mg⁻¹ protein was determined.

3 Correlation of antagonist affinities against [³H]-rauwolscine with corresponding values in the other four tissue sources showed the Y79 cells to resemble most closely the OK cells, the prototype example of an α_{2C} -adrenoceptor, with a correlation coefficient of 0.90 and a regression slope of 1.01 being obtained for 10 antagonists in these two systems.

4 Comparison of K_D values for [³H]-rauwolscine also showed a similarity between the OK cells (0.19 ± 0.07 nM) and Y79 cells.

5 These data suggest that the human retinoblastoma cell line may represent an additional example of the α_{2C} -adrenoceptor subtype.

Keywords: HT29 cells; NG108-15 cells; rat sublingual gland; [³H]-rauwolscine; α_2 -adrenoceptor subtypes

Introduction

In the past few years, several groups have succeeded in cloning and expressing α_2 -adrenoceptors from rat, human and porcine sources (Lorenz *et al.*, 1990; Guyer *et al.*, 1990; Weinshank *et al.*, 1990; Flordellis *et al.*, 1991; Harrison *et al.*, 1991; Chhajlani *et al.*, 1991; Voigt *et al.*, 1991; Lanier *et al.*, 1991). From these molecular biological studies, it is clear that there are at least three α_2 -adrenoceptor subtypes, having distinct amino acid sequences. However, it is not yet clear whether the three α_2 -adrenoceptor proteins isolated from rat correspond to the three isolated from human sources (Harrison *et al.*, 1991) or whether a fourth subtype has been cloned from the rat genomic library (Lanier *et al.*, 1991). Likewise, assignment of the cloned receptor proteins to the multiple α_2 -adrenoceptor subtypes which have been identified by radioligand binding assays (Bylund, 1988; Uhlen & Wikberg, 1991) is still controversial.

Binding sites defined as α_2 -adrenoceptors, based on their high affinity for [³H]-rauwolscine, can be unequivocally subclassified into the α_{2A} - and α_{2B} -adrenoceptor subgroups, based on high (α_{2B}) or low (α_{2A}) affinity for prazosin or ARC-239. Multiple examples of both α_{2A} - and α_{2B} -adrenoceptors are known, both in tissue culture lines and in tissue homogenates from several species. Functional studies measuring the ability of prazosin and ARC-239 to inhibit adenylate cyclase activation by an α_2 -adrenoceptor agonist in HT29 (α_{2A}) and NG108-15 (α_{2B}) cells support this subclassification scheme (Bylund & Ray-Prenger, 1989). Cloned and expressed α_2 -adrenoceptor proteins can also be divided into two groups, having either high (<100 nM) or low (>1000 nM) affinity for prazosin (Simmoneaux *et al.*, 1991; Harrison *et al.*, 1991; Lanier *et al.*, 1991).

When the ability of a diverse series of antagonists to inhibit [³H]-rauwolscine binding was compared in a variety of tissues, it became apparent that there were more than two α_2 -adrenoceptor binding sites. It is currently thought that at least four α_2 -adrenoceptors can be identified by this technique (Bylund, 1988; Blaxall *et al.*, 1991; Simmoneaux *et al.*, 1991). The two additional sites have been designated as α_{2C} and α_{2D} . Although the α_{2C} and α_{2D} sites resemble the α_{2B} and α_{2A} , respectively, antagonists capable of differentiating the four sites are available (Blaxall *et al.*, 1991). By examining the simultaneous interactions of several antagonists at the α_2 -adrenoceptor binding site it may be possible to identify even more discrete binding sites (Uhlen & Wikberg, 1991).

The α_{2C} -adrenoceptor has only been identified thus far in the OK cell line, derived from the opossum kidney, and in tissue homogenates prepared from fresh opossum kidneys (Blaxall *et al.*, 1991), and it has been suggested that the α_{2C} and α_{2B} -adrenoceptors might represent species variants (Lorenz *et al.*, 1990). Hence the demonstration of an α_{2C} site in another tissue source, not derived from the opossum, would strengthen the case for its role as a distinct α_2 -adrenoceptor subtype. In this report, we compare the ability of a series of 10 antagonists to inhibit the binding of [³H]-rauwolscine to membrane homogenates from five tissues/cell lines. Tissues examined include known examples of α_{2A} (HT29 cells), α_{2B} (NG108-15 cells), α_{2C} (OK cells) and α_{2D} (rat sublingual gland), as well as a human derived retinoblastoma cell line (Y79; Reid *et al.*, 1974). Y79 cells have been shown to have α_2 -adrenoceptors, which were suggested to be of the α_{2A} subtype (Kazmi & Mishra, 1989).

Methods

Cell culture

HT29 cells (American Type Culture Collection (ATCC), Rockville, MD, U.S.A.) and OK cells (kindly donated by Dr

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D.B. Bylund, University of Nebraska, Omaha, NB, U.S.A.) were grown in Dulbecco's modified Eagle's medium (DMEM) containing high glucose and supplemented with 10% foetal bovine serum (FBS). NG108-15 cells (kindly donated by Dr Marshall Nirenberg, National Institutes of Health, Bethesda, MD, U.S.A.) were grown in DMEM supplemented with 10% FBS, 0.1 mM hypoxanthine, 0.4 μ M aminopterin and 16 μ M thymidine. Y79 cells (ATCC) were grown in suspension in RPMI 1640 medium supplemented with 15% FBS. To obtain cells for tissue preparation, HT29 and OK cells were subcultured with 0.05% trypsin containing 0.53 mM ethylenediaminetetraacetic acid (EDTA), and NG108-15 cells were gently agitated loose from the culture flask. Cells were maintained in a humidified atmosphere consisting of 5% CO₂: 95% air (10% CO₂:90% air for NG108-15) and harvested for membrane preparation after reaching confluence.

Tissue preparation

Cells were scraped, pelleted by centrifugation (300 g, 5 min) and washed twice in Dulbecco's phosphate buffered saline (composition in mM: KCl 2.7, KH₂PO₄ 1.5, NaCl 137 and Na₂HPO₄ 8.1). The pH of this solution was 7.2. The cell pellet was resuspended in ice-cold 5.0 mM Tris, 1.0 mM EDTA, pH 7.4, and homogenized on ice with a polytron (Brinkman, Westbury, NY, U.S.A.) (3 $\times$ 15 s burst on setting 6). The homogenate was centrifuged (500 g, 5 min). The supernatant was collected and centrifuged at 100,000 g for 30 min. The resulting membrane pellet was resuspended in 25 mM glycylglycine, pH 7.6, and frozen at -70°C following protein determination.

Rat sublingual glands were dissected free from the submaxillary gland (Pel Freeze, Rogers, AK, U.S.A.) and homogenized as above in ice-cold homogenizing buffer containing (mM): sucrose 250; Tris 10, EDTA 0.002 at pH 7.5 until a uniform homogenate was observed. Membranes were then prepared and stored as described above.

Protein content was determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA, U.S.A.) using the method described by Bradford (1976) with bovine serum albumin as standard.

Radioligand binding assay

All binding assays were incubated in 25 mM glycylglycine (Bylund, 1985), at 25°C for 30 min. Binding was stopped by vacuum filtration using a Brandell cell harvester through Whatman GF/B filters, with three 5 ml washes with ice-cold binding buffer. Radioactivity retained on the filter was determined by liquid scintillation counting in ReadySafe scintillation fluid.

For [³H]-rauwolscine saturation studies, total binding was determined in duplicate tubes containing a predetermined concentration of the radioligand and 100–250 μ g membrane protein in a total volume of 300 μ l. Nonspecific binding was determined in the presence of 10 μ M phentolamine. Specific binding was calculated as the difference between total and nonspecific binding. The K_D and B_{max} were calculated from nonlinear regression with the aid of the AccuFit computer programme (Beckman, Fullerton, CA, U.S.A.).

For competition studies, various concentrations of α -adrenoceptor agonists and antagonists were incubated in the presence of the K_D of [³H]-rauwolscine for the tissue source being examined and 100–250 μ g membrane protein in a total volume of 300 μ l. K_i values were determined from nonlinear regression by the Lundo₂ computer programme (Lundon Software Inc., Cleveland, OH, U.S.A.).

Drugs and solutions

SK&F 86466 (6-chloro-2, 3, 4, 5-tetrahydro-3-methyl-1H-3-benzazepine), SK&F 104078 (6-chloro-9- [(3-methyl-2-butenyl) oxy]-2,3,4,5-tetrahydro-3-methyl-1H-3-benzazepine),

SK&F 104856 (2-vinyl-7-chloro-3,4,5,6-tetrahydro-4-methylthieno [4,3,2ef][3] benzazepine and BRL 44408 (2-[(4, 5-dihydro-1H-imidazol-2-yl) methyl]-2, 3-dihydro-1-methyl-1H-isoindole) were synthesized by the Medical Chemistry Department of SmithKline Beecham (King of Prussia, PA, U.S.A. or Great Burgh, Surrey, U.K.) BAM1303 (8 β [2-phenylimidazol-1-yl)methyl-6-methylergoline; Maruko Pharmaceutical Co. Ltd., Nagoya, Japan), ARC-239 (2-[2,4-(*o*-methoxyphenyl)piperazin-1-yl]ethyl-4,4-dimethyl-1,3- (2H,4H)-isoquinolindione; Karl Thomae, Biberach, Germany) and prazosin (Pfizer, Groton, CT, U.S.A.) were kindly donated by their manufacturers. Sources of reagents were as follows: GIBCO, ST. Louis, MO, U.S.A. (trypsin, DMEM, DPBS, FBS); Dupont New England Nuclear, Wilmington, DE, U.S.A. ([³H]-rauwolscine, 78–86 Ci mmol⁻¹); CIBA, Summit NJ, U.S.A. (phentolamine); Reseach Organics, Cleveland, OH, U.S.A. (glycylglycine); Karl Roth, Karlsruhe, Germany (rauwolscine). All other compounds and reagents were obtained from Sigma, St. Louis, MO, U.S.A.

All compounds were dissolved in the appropriate buffer with the exception of ARC 239 which was dissolved in 0.1 N HCl and immediately diluted in buffer, and BAM 1303 which was dissolved in ethanol. The maximal ethanol concentration in the assay medium was 1%. This concentration was shown to have no effect on the binding of [³H]-rauwolscine.

Results

Representative saturation curves and Scatchard plots for [³H]-rauwolscine in Y79 cells and rat sublingual gland are shown in Figure 1. Although characterization of α_2 -adrenoceptors has previously been reported in rat submaxillary gland (Michel *et al.*, 1989), we found that when the sublingual gland was separated by dissection from the total 'submaxillary gland' obtained from the supplier, essentially all of the specific [³H]-rauwolscine binding was to the sublingual component (data not shown). This may be reflected in our higher B_{max} value (301 fmol mg⁻¹ protein; Table 1) compared to the value obtained by Michel *et al.* (1989) in the total submaxillary gland (137 fmol mg⁻¹ protein). K_D and B_{max} values for [³H]-rauwolscine binding to HT29, HG108-15 and OK cells are also presented in Table 1. From Table 1 it is apparent that (1) the receptor density in the Y79 cells is substantially lower than that in the other systems and (2) the affinity of [³H]-rauwolscine for the binding sites in OK and Y79 cells is higher than in the other preparations. In all systems examined the specific binding of [³H]-rauwolscine represented at least 70% of the total binding to the tissue homogenate.

The potency of a series of 10 compounds in inhibiting the binding of [³H]-rauwolscine to the five tissue sources is shown in Table 2. The compounds examined included eight antagonists, plus the partial agonists, oxymetazoline and clonidine. The selectivity previously reported for prazosin, ARC 239 and SK&F 104856 in NG108-15 versus HT29 cells; and, conversely, oxymetazoline and BRL 44408 in HT29 versus NG108-15 cells is confirmed by our data. Consistent with the K_D values for [³H]-rauwolscine as determined by saturation analysis, the K_i for unlabelled rauwolscine was lower in the OK and Y79 cells.

Correlation of drug affinities as inhibitors of [³H]-rauwolscine binding to the five tissue sources shows a statistically significant relationship only for HT29 cells versus sublingual gland and for Y79 cells versus OK cells (Table 3).

Discussion

Our data provide additional support for the premise that α_2 -adrenoceptors can be subdivided based on the ability of α -adrenoceptor agonists and antagonists to displace [³H]-rauwolscine binding. As previously reported (Bylund, 1988)

the [3 H]-rauwolscine sites on HT29, NG108-15 and OK cells clearly represent three distinct groups. In agreement with Blaxall *et al.* (1991) we found the affinity of [3 H]-rauwolscine to be highest in the OK cells, although our calculated affinity (as reflected by the K_D for [3 H]-rauwolscine or the K_i for unlabelled rauwolscine as an inhibitor of binding) was about five fold lower than that reported by these investigators. Comparison of our data in the OK and HT29 cells with that of Blaxall *et al.* (1991) shows, with the exception of oxymetazoline and SK&F 104078 in the OK cell line, our K_i values to

Figure 1 Representative experiments showing saturation curves and Scatchard analyses (inset) for binding of [3 H]-rauwolscine to membrane homogenates of rat sublingual gland (a) and Y79 cells (human retinoblastoma; b). Each point represents the mean of duplicate determinations.

Table 1 Binding parameters of [3 H]-rauwolscine

Tissue	K_d (nM)	B_{max} (fmol mg ⁻¹ protein)	SB^a
HT29 cells (α_{2A})	1.10 ± 0.45	170 ± 7	87
NG108-15 cells (α_{2B})	1.37 ± 0.06	235 ± 20	81
OK cells (α_{2C})	0.19 ± 0.07	245 ± 27	93
Rat sublingual gland (α_{2D})	5.04 ± 0.37	301 ± 15	69
Y79 cells	0.30 ± 0.10	38 ± 1	73

^aSpecific binding as % of total binding.

Values represent mean $\pm$ s.e.mean, derived from at least 2 experiments, each performed in duplicate.

be 3 to 5 fold higher, suggesting the difference may be due to slight changes in experimental methodology. Nevertheless, the relative potencies in these two cell lines of the antagonists tested both by us and Blaxall *et al.* are nearly identical.

We found the α_2 -adrenoceptor on the rat sublingual gland to be distinct from that on the NG108-15 and OK cells, but not from that on the HT29 (Table 3). Other studies have shown that the cross-subtype correlation for inhibition of [3 H]-rauwolscine binding is closest ($r > 0.8$ with regression slopes near unity) when α_{2D} (bovine pineal, rat submaxillary gland) are compared to α_{2A} (HT29 cells, chicken pineal) systems (Simmoneaux *et al.*, 1991). Furthermore, most of the agents examined in our study do not discriminate clearly between α_{2A} - and α_{2D} -adrenoceptors. Of the systems we examined, the affinity of rauwolscine is lowest for the receptor of the sublingual gland, as reflected either by the K_D (5 nM) or K_i (11 nM). This relatively low affinity of rauwolscine is characteristic of an α_{2D} subtype (Simmoneaux *et al.*, 1991).

Our data in the Y79 cells agrees closely with that reported by Kazmi & Mishra (1979). Although they assign the α_2 -adrenoceptor in this cell line to the α_{2A} subtype, this assignment was based only on the relative potencies of prazosin and oxymetazoline. When our K_i values in the Y79 cells are compared to the corresponding values in a known α_{2A} system, the HT29 cells, the correlation is poor ($r = 0.57$, Table 3). Although the overall correlation of the Y79 and NG108-15 data is slightly better ($r = 0.65$), the three antagonists known to be selective for the α_{2B} subtype, prazosin, ARC239 and SK&F 104856 (Gleason & Hieble, 1991), all are significantly less potent in the Y79 system (Table 2). An excellent correlation is obtained when K_i values in Y79 and OK cells are compared ($r = 0.9$; Table 3). Furthermore, the best-fit regression and line of identity between these two systems are superimposable.

It is somewhat disturbing that the only compound deviating from the correlation is BAM1303, reported to be one of the most selective antagonists for α_{2C} -adrenoceptors (Blaxall

Table 2 Potency of drugs as inhibitors of [3 H]-rauwolscine binding

	K_i (nM) ^a				
	HT29 ^b	NG108-15 ^b	OK	Rat sublingual	Y79
ARC 239	466 ± 44	9.62 ± 0.81	41.3 ± 5.3	420 ± 84	60.2 ± 17.9
BAM 1303	12.7 ± 3.8	49.5 ± 6.4	1.98 ± 0.42	86.2 ± 25.2	14.8 ± 5.9
Clonidine	16.4 ± 1.4	21.8 ± 2.2	24.5 ± 8.2	10.3 ± 2.5	10.2 ± 3.7
Oxymetazoline	2.07 ± 0.76	225 ± 111	36.3 ± 11.3	11.5 ± 3.3	19.5 ± 8.1
Prazosin	779 ± 88	22.5 ± 10.6	79.9 ± 4.4	175 ± 38	125 ± 29
Rauwolscine	2.01 ± 0.66	1.47 ± 0.20	0.26 ± 0.03	11.1 ± 3.0	0.54 ± 0.24
BRL 44408	5.35 ± 2.85	205 ± 40	187 ± 24	15.7 ± 2.3	227 ± 78
SKF 86466	8.69 ± 2.01	4.91 ± 0.87	8.28 ± 1.11	5.17 ± 1.59	3.96 ± 0.53
SKF 104078	105 ± 29	71.5 ± 9.3	58.6 ± 6.7	202 ± 53	47.2 ± 7.3
SKF 104856	257 ± 52	7.77 ± 0.99	59.6 ± 19.1	117 ± 33	42.8 ± 9.9

^aValues are mean $\pm$ s.e.mean and are derived from at least 3 experiments performed in duplicate. ^bData from Gleason & Hieble (1991).

Table 3 Correlation of inhibitory potency between tissues

Tissue	r	Slope	P
HT29 cells vs NG108-15 cells (α_{2A} vs α_{2B})	0.07	0.09	<0.841
HT29 cells vs OK cells (α_{2A} vs α_{2C})	0.44	0.49	<0.173
HT29 cells vs Sublingual gland (α_{2A} vs α_{2D})	0.84	1.09	<0.001
NG108-15 cells vs OK cells (α_{2B} vs α_{2C})	0.47	0.41	<0.141
NG108-15 vs Sublingual gland (α_{2B} vs α_{2D})	0.18	0.18	<0.598
OK cells vs Sublingual gland (α_{2C} vs α_{2D})	0.22	0.25	<0.524
HT29 cells vs Y79 cells	0.57	0.72	<0.086
NG108-15 cells vs Y79 cells	0.65	0.60	<0.044
OK cells vs Y79 cells	0.90	1.01	<0.0001
Sublingual gland vs Y79 cells	0.56	0.50	<0.095

Values are derived from linear regression analysis of the correlations of the log of the drug affinities presented in Table 2.

et al., 1991). Nevertheless, in addition to the excellent overall correlation of K_i values, the OK and Y79 cells share several characteristics distinguishing them from the other systems studied: (1) a 5–10 fold lower potency of ARC 239, prazosin and SK&F 104856 *vis-a-vis* the NG-108 cells; this difference is consistent with the relative potencies for these antagonists in the OK cells compared with another α_{2B} system, the neonatal rat lung (Simmoneaux *et al.*, 1991). (2) A higher affinity for rauwolscine (3–20 fold difference for the Y79 cells compared with the NG-108 15, HT29 or rat sublingual preparations). (3) An oxymetazoline affinity intermediate between that observed in the α_{2A} and α_{2B} models. Hence, we propose that the Y79 cell line represents an additional example of an

α_{2C} -adrenoceptor.

Based on the ability of mianserin enantiomers to block α_2 -adrenoceptor-mediated inhibition of synaptosomal nor-adrenaline release (Raiteri *et al.*, 1983), it has been postulated that a functional α_{2C} -adrenoceptor may be present in the rat cerebral cortex (Blaxall *et al.*, 1991). Our results, showing these receptors to be present in a cell line derived from a human tumour (Reid *et al.*, 1974) provide further support for the premise that the α_{2C} -adrenoceptor is not limited to the opossum. Further studies, using the selective antagonists now available, will be required to establish the distribution and functional role of this receptor subtype.

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