Pharmacological properties of the cloned $\alpha_{1A/D}$ -adrenoceptor subtype are consistent with the α_{1A} -adrenoceptor characterized in rat cerebral cortex and vas deferens

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1 The pharmacological characteristics of cloned mammalian $\alpha_{1A/D}$ -, α_{1B} - and α_{1C} -adrenoceptor subtypes expressed in rat 1 fibroblasts were determined in comparison to the binding and functional properties of these subtypes in rat tissues.

2 Analysis of [³H]-prazosin binding to membrane homogenates from rat 1 fibroblast cells expressing each of the α_1 -subtypes indicated high affinity binding to a single population of binding sites. Binding affinities were similar for $\alpha_{1A/D}$ -, α_{1B} - and α_{1C} -subtypes (K_d s: 0.13, 0.10 and 0.15 nM respectively) although a higher density of α_{1B} - and α_{1C} -receptors (B_{max} : 4068 and 10,323 fmol mg⁻¹ protein respectively) were expressed in comparison to $\alpha_{1A/D}$ (838 fmol mg⁻¹).

3 Displacement of [³H]-prazosin from membranes expressing cloned α_1 -adrenoceptor subtypes revealed that 5-methyl-urapidil, WB 4101, benoxathian and phentolamine displayed high affinity and selectivity for $\alpha_{1A/D}$ - over α_{1B} -subtypes. These compounds also had high affinity and selectivity for α_{1C} - over α_{1B} -subtypes. 5-Methyl-urapidil showed selectivity for α_{1C} (K_i 0.60 ± 0.16 nM) over both $\alpha_{1A/D}$ (K_i 9.8 ± 2.8 nM) and α_{1B} (K_i 57.2 ± 12 nM) subtypes. Prazosin and doxazosin were not subtype selective.

4 In comparison to [³H]-prazosin a similar pharmacological profile was obtained with [¹²⁵I]-HEAT using cloned $\alpha_{1A/D}$ -, α_{1B} - and α_{1C} -adrenoceptors expressed in rat 1 fibroblasts.

5 The affinities of prazosin, WB 4101, 5-methyl-urapidil, phentolamine and benoxathian at cloned $\alpha_{1A/D}$ -receptors were consistent with α_{1A} affinities determined with chlorethylclonidine-treated rat cortical membranes. Affinities at cloned α_{1B} -receptors were consistent with α_{1B} affinities determined with rat liver membranes.

6 Using the epididymal rat vas deferens as a functional measure of α_{1A} affinity, prazosin (pA_2 9.23 ± 0.28), WB 4101 (pA_2 9.58 ± 0.12), phentolamine (pK_B 7.90 ± 0.16), benoxathian (pK_B 9.21 ± 0.21) and 5-methyl-urapidil (pK_B 8.51 ± 0.16) were potent antagonists of noradrenaline-induced contractions.

7 At present, evidence from cloning studies suggests the existence of at least three α_1 -adrenoceptor subtypes. In contrast to the recent proposal for α_1 -adrenoceptor classification, the pharmacology of the cloned $\alpha_{1A/D}$ (or α_{1D})-adrenoceptor is more consistent with that of an α_{1A} -adrenoceptor characterized in rat cerebral cortex and vas deferens.

Keywords: α_1 -Adrenoceptors; cloned receptors; rat vas deferens; rat cortex

Introduction

Heterogeneity amongst α_1 -adrenoceptors has been evident for nearly a decade on the basis of functional responses in smooth muscle (see McGrath *et al.*, 1989). The first definitive classification of α_1 -subtypes, established by use of radioligand binding, was proposed by Morrow & Creese (1986) based on the ability of certain antagonists such as WB 4101 and phentolamine to discriminate between high and low affinity binding sites in rat cerebral cortex. Further characterization with the alkylating agent chlorethylclonidine (CEC) indicated that the low affinity sites for WB 4101 were also sensitive to CEC and were designated α_{1B} , the high affinity sites being α_{1A} (Minneman *et al.*, 1988; Bylund, 1992). Both α_{1A} - and α_{1B} -subtypes have different anatomical distributions and appear to be differentially coupled to the mobilisation of extra and intracellular calcium respectively (Han *et al.*, 1987; Minneman *et al.*, 1988; Lomasney *et al.*, 1991). In addition to WB 4101, binding and functional studies have indicated that several compounds including 5-methyl urapidil and niguldipine have selectivity for α_{1A} - over α_{1B} -subtypes (Hanft & Gross, 1989; Gross *et al.*, 1989; Minneman & Atkinson, 1991). Evidence for the existence of further α_1 -subtypes has

largely been substantiated by molecular cloning studies. The first subtype to be identified was the α_{1B} -adrenoceptor from the hamster DDT1-MF2 smooth muscle cell line (Cotecchia *et al.*, 1988), followed by the identification of a novel α_1 -subtype from a bovine cDNA library which has been termed α_{1C} (Schwinn *et al.*, 1990; 1991). Both subtypes are pharmacologically distinct but share a common signalling mechanism in that both α_{1B} and α_{1C} -subtypes mobilize intracellular Ca²⁺ through the activation of phospholipase C via a pertussis toxin-insensitive G protein (Schwinn *et al.*, 1991). Using an oligonucleotide probe based on the cDNA of the hamster α_1 -subtype, screening a rat cerebral cortex cDNA library then identified two further clones (Lomasney *et al.*, 1991). The first proved to be the rat homologue of the hamster α_{1B} -adrenoceptor whilst the second was claimed to represent the first identification of the putative α_{1A} subtype on the basis of insensitivity to CEC, high affinity for WB 4101 and phentolamine and tissue distribution in rat tissues: thus, Northern analysis with an α_{1A} -receptor cDNA probe detected high levels in rat vas deferens, hippocampus and cortex.

A putative fourth α_1 -subtype, also identified from a rat brain cDNA library, was described by Perez *et al.* (1991). Initially this subtype was claimed to be distinct from the α_{1A} subtype and termed α_{1D} on the basis of its pharmacological

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profile and differential sensitivity to CEC. However, it is now believed that these clones code for the same α_1 subtype (differing by only two codons in sequence) and it has recently been proposed that this subtype be designated $\alpha_{1A/D}$ (Schwinn & Lomasney, 1992). This classification has been proposed because of the low affinities shown by several α_1 antagonists for the cloned $\alpha_{1A/D}$ -subtype in comparison to the potency and pharmacological profile of these compounds at the α_{1A} -receptor defined in tissue binding and functional experiments (Schwinn & Lomasney, 1992). This therefore suggested the existence of a further α_1 -subtype, yet to be identified in molecular terms, but corresponding to the tissue α_{1A} -subtype.

In this paper, we have characterized the pharmacological profile of a variety of compounds at cloned $\alpha_{1A/D}$, α_{1B} and α_{1C} -subtypes in conjunction with functional and binding studies. Our data indicate that the pharmacological properties of the cloned $\alpha_{1A/D}$ -adrenoceptor are entirely consistent with the α_{1A} -subtype characterized in rat cerebral cortex and vas deferens.

Methods

Expression of cloned α_1 -adrenoceptor subtypes

Construction and transfection of rat $\alpha_{1A/D}$, hamster α_{1B} and bovine α_{1C} -adrenoceptors was carried out at Duke University, Durham, N.C. U.S.A. by use of methods described by Allen *et al.* (1991). Cloned receptors expressed in rat 1 fibroblasts were subsequently obtained commercially through TULCO (Research Triangle Park, N.C., U.S.A.). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum and 300 $\mu\text{g ml}^{-1}$ G418 sulphate, a neomycin analogue. For subculturing, cell monolayers were washed with Hank's balanced salt solution and trypsinized briefly with 0.05% trypsin, 0.5 mM EDTA and split 1:5 to 1:20 every 3–4 days.

Radioligand binding assays

Radioligand binding experiments were performed with membranes prepared from rat 1 fibroblast cells expressing individual α_1 -subtypes or from rat tissues as indicated. Scraped cells or dissected tissues were homogenized in ice cold 50 mM Tris buffer (pH 7.5) in a Polytron homogenizer (PT10, setting 6, 20 s). The membranes were washed three times by centrifugation (20 min at 20,000 g) and resuspended in fresh buffer before storage at -70°C . For pretreatment with chlorethyl-clonidine, membranes were incubated at 37°C for 15 min in 50 mM Tris buffer (pH 7.5) in the presence of 10 μM CEC followed by three further washes in ice cold 50 mM Tris buffer before storage. Tissue membranes were stored at a concentration of 2.5 mg ml^{-1} protein, cell membranes at 0.5 mg ml^{-1} protein. Binding of [^{125}I]-HEAT was carried out as described by Schwinn & Lomasney (1992). Binding of [^3H]-prazosin was measured in 400 μl aliquots of diluted membranes (200 μg tissue protein or 10 μg fibroblast protein) in 50 mM Tris buffer (pH 7.5) in a final assay volume of 500 μl . Non specific binding was determined in the presence of 1 μM phentolamine. Construction of saturation isotherms was determined by sequential dilution from a final concentration of 8 nM [^3H]-prazosin. Assays were incubated at 25°C with [^3H]-prazosin for 30 min and for 45 min with [^{125}I]-HEAT. All reactions were terminated by the addition of ice cold Tris buffer and rapid cold filtration over Whatman GF/B filters under vacuum. Saturation and displacement binding data were analysed by iterative non-linear curve fitting programmes.

Epididymal rat vas deferens

Vas deferens were removed from male Charles River Caesarian derived rats (250–320 g). The epididymal portions were

mounted in 15 ml organ baths containing Krebs buffer of the following composition (mM): NaCl 120, NaHCO_3 25, glucose 11, KCl 4.7, KH_2PO_4 1.2, MgSO_4 1.2, CaCl_2 2.5 containing cocaine 10 μM and corticosterone 10 μM . The medium was maintained at 37°C , pH 7.4 and aerated with a 95% O_2 /5% CO_2 mixture. A resting tension of 0.5 g was applied and changes in isometric tension measured via force-displacement transducers. The preparations were equilibrated for 60 min before any additions to the bath. Each tissue was exposed to a sensitizing dose of 100 μM noradrenaline (NA) for 1 min and then washed periodically for 30 min. Concentration-response curves (CRC) were constructed by non-cumulative addition of NA in 0.5 log increments from 0.01 μM to a maximum of 100 μM with a 10 min wash period between each dose. The preparations were then equilibrated with or without antagonist for 30 min before a second CRC to NA. Antagonist pA_2 values were obtained from a plot of log (agonist $\text{DR} - 1$) against log antagonist concentration where the slope was not different from unity (Arunlakshana & Schild, 1959). K_B (antagonist dissociation constant) was determined from the equation $K_B = [A]/(\text{DR} - 1)$ where the dose ratio (DR) was produced by a single concentration of antagonist [A].

Drugs used in the study

The following drugs (sources in parentheses) were used: [^3H]-prazosin and [^{125}I]-HEAT (2- β -(4-hydroxy-3- [^{125}I]-iodophenyl)ethylaminomethyl)-tetralone) (Amersham, U.K.); noradrenaline, cocaine hydrochloride, and corticosterone (Sigma, U.K.); chlorethylclonidine, spiperone, benoxathian, 5-methylurapidil, WB 4101 (2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4-benzodioxane hydrochloride (Research Biochemicals Inc., Semat, U.K.); phentolamine hydrochloride (Ciba-Geigy, Basle, Switzerland); prazosin and doxazosin (Pfizer, Sandwich, U.K.). All other drugs and chemicals were obtained from Sigma (U.K.) or B.D.H. (U.K.). Drugs were dissolved in distilled H_2O or dimethyl sulphoxide (DMSO) at 1 mM and subsequent dilutions made in assay buffer.

Results

[^3H]-prazosin binding to cloned mammalian α_1 -adrenoceptor subtypes

Analysis of [^3H]-prazosin binding to membranes prepared from rat 1 fibroblasts expressing each of the three α_1 subtypes indicated saturable, high affinity binding to a single population of receptor sites. The affinity of [^3H]-prazosin binding sites was similar for all subtypes, although a high density of α_{1B} - and α_{1C} -receptors was expressed in rat 1 fibroblasts in comparison to $\alpha_{1A/D}$ (Table 1). The non-selective antagonists, doxazosin and prazosin, displayed high and similar affinity at all three cloned α_1 -subtypes and, as with all other compounds examined, displaced the ligand with Hill slopes not significantly different from unity, indicating an apparently competitive interaction at a single population of binding sites (Table 2). However, several compounds displayed differing affinities for the three subtypes having selectivity for one or more of the cloned α_1 -subtypes. WB 4101, 5-methylurapidil, benoxathian and phentolamine showed high affinity and selectivity for cloned $\alpha_{1A/D}$ -over α_{1B} -adrenoceptor subtypes. However, these agents also showed varying degrees of selectivity for α_{1C} over $\alpha_{1A/D}$ (Table 2) and in this respect, 5-methylurapidil was highly selective for α_{1C} - over both $\alpha_{1A/D}$ - and α_{1B} -subtypes.

[^3H]-prazosin binding to rat liver and CEC-treated cortical membranes

In this series of experiments we considered it important to establish conditions with CEC which were appropriate and

Table 1 Saturation binding parameters for [3 H]-prazosin at cloned α_1 -adrenoceptor subtypes

	$\alpha_{1A/D}$	α_{1B}	α_{1C}
K_d (nM)	0.13 $\pm$ 0.02	0.10 $\pm$ 0.01	0.15 $\pm$ 0.02
B_{max} (fmol mg $^{-1}$)	838 $\pm$ 46	4068 $\pm$ 342	10323 $\pm$ 974

Saturation binding parameters were determined with [3 H]-prazosin (0.01–8.0 nM) using membrane homogenates from rat 1 fibroblast cells expressing each of the α_1 -subtypes. Data represent the mean $\pm$ s.e.mean of 9–10 experiments carried out in duplicate.

Table 2 Comparative affinities of α_1 -adrenoceptor antagonists for cloned $\alpha_{1A/D}$ -, α_{1B} - and α_{1C} -adrenoceptor subtypes

Compound (n)	K_i (nM)		
	$\alpha_{1A/D}$	α_{1B}	α_{1C}
Prazosin (18)	0.32 $\pm$ 0.02	0.19 $\pm$ 0.02	0.27 $\pm$ 0.04
Doxazosin (6)	1.99 $\pm$ 0.05	0.80 $\pm$ 0.02	1.58 $\pm$ 0.10
WB 4101 (14)	0.67 $\pm$ 0.07	16.4 $\pm$ 2.0	0.29 $\pm$ 0.03
5-Methyl urapidil (7)	9.8 $\pm$ 2.8	57.2 $\pm$ 12	0.60 $\pm$ 0.16
Phentolamine (10)	6.1 $\pm$ 0.9	39.8 $\pm$ 4.3	2.8 $\pm$ 0.3
Benoxathian (6)	0.50 $\pm$ 0.02	17.2 $\pm$ 4.4	0.30 $\pm$ 0.05
Spiperone (4)	25.6 $\pm$ 3.2	6.8 $\pm$ 1.0	7.5 $\pm$ 0.9

Displacement of [3 H]-prazosin (0.2 nM) from rat 1 fibroblast membranes expressing each of the α_1 -subtypes was determined with at least 10 concentrations of competing drug. Assay points were determined in duplicate. For all compounds examined Hill slopes (nH) were not significantly different from unity.

Table 3 The effect of chlorethylclonidine (CEC) on [3 H]-prazosin saturation binding to rat cortical and liver membranes

Time	Control		+ CEC	
	K_d	B_{max}	K_d	B_{max}
Rat cortex	0.20 $\pm$ 0.02	251 $\pm$ 27	0.22 $\pm$ 0.03	130 $\pm$ 19
Rat liver	0.32 $\pm$ 0.04	508 $\pm$ 31	0.28 $\pm$ 0.02	43 $\pm$ 9

Membranes were incubated with CEC (10 μ M) for 15 min at 37°C. Saturation parameters K_d (nM) and B_{max} (fmol mg $^{-1}$ protein) were determined by iterative curve fitting. Values represent the mean $\pm$ s.e.mean of 3–4 experiments.

Figure 1 Inhibition of [3 H]-prazosin binding to rat cortical membranes by 5-methyl-urapidil using control membranes (O) or membranes pretreated with chlorethylclonidine (CEC) 10 μ M for 15 min at 37°C (●). In control membranes the data were best described by a two site fit (site 1, 51.1%, IC_{50} = 632 nM and site 2, 48.9%, IC_{50} = 3 nM). Using CEC-treated membranes the data were best described by a one site fit (IC_{50} = 2.2 nM, nH = 0.92). Data from a representative experiment are shown, calculated mean K_i values are given in the text.

selective for the alkylation of a given population of adrenoceptors prior to the interpretation of [3 H]-prazosin data from CEC-treated cortical membranes containing a heterogeneous receptor population. Pretreatment of membrane homogenates with CEC (15 min at 37°C) resulted in alkylation of more than 90% of the sites in rat liver and 49% of the sites in rat cortex (Table 3).

In rat liver membranes, the affinity and selectivity of all compounds examined (prazosin > WB 4101 > benoxathian > phentolamine > 5-methyl-urapidil) was similar to that observed at cloned α_{1B} -subtypes (Table 4). Taken together with the sensitivity of the membrane preparations to CEC, these data indicated the presence of α_{1B} -receptors in rat liver. Under conditions with CEC which resulted in alkylation of 90% of the α_1 population in rat liver membranes (Table 3), we then examined the properties of the remaining α_1 population in rat brain membranes following a similar CEC protocol (Figure 1). Table 4 illustrates that under this protocol, the α_1 population remaining in rat brain following CEC treatment retained high affinity for all compounds examined. Furthermore, the affinities obtained were similar to those observed at cloned $\alpha_{1A/D}$ -subtypes indicating a similar pharmacological profile of rat cortical and cloned $\alpha_{1A/D}$ -adrenoceptors.

[125 I]-HEAT binding to cloned α_1 -adrenoceptor subtypes

The pharmacological profile of cloned α_1 -adrenoceptors labelled with [125 I]-HEAT was examined with a range of α_1 -adrenoceptor antagonists (Table 5). Similar to the profile obtained with [3 H]-prazosin, WB 4101, benoxathian and 5-methyl-urapidil appeared to be selective for $\alpha_{1A/D}$ over α_{1B} . The rank order of compounds was similar to that observed with [3 H]-prazosin, except that phentolamine displayed slightly higher affinity for $\alpha_{1A/D}$ sites labelled by [125 I]-HEAT. Similarly, at cloned α_{1C} -adrenoceptors, whilst the rank order of potency for compounds was similar with both ligands, slightly lower potency was observed against [125 I]-HEAT and thus spiperone and 5-methyl-urapidil appeared to be less selective for α_{1C} over $\alpha_{1A/D}$ than observed with [3 H]-prazosin.

Effects of α_1 -adrenoceptor antagonists on contractile responses of epididymal rat vas deferens

Noradrenaline (0.01–100 μ M) produced concentration-dependent contractions of epididymal rat vas deferens generating a maximal tension of 2.24 $\pm$ 0.12 g with an EC_{50} of 0.10 $\pm$ 0.65 μ M (n = 10). Prazosin and WB 4101 antagonized NA-induced contractions and shifted control CRC to the right in parallel which yielded pA_2 values of 9.23 $\pm$ 0.28 (slope = 1.2) and 9.58 $\pm$ 0.12 (slope = 1.1) respectively (Figure 2). Estimated pK_B values (antagonists at 100 nM) were calculated to be 9.21 $\pm$ 0.21, 7.90 $\pm$ 0.11 and 8.51 $\pm$ 0.16 for benoxathian, phentolamine and 5-methyl-urapidil respectively.

Discussion

In this paper we have examined the pharmacological profile of cloned mammalian $\alpha_{1A/D}$ -, α_{1B} - and α_{1C} -subtypes. We have compared these data with the profile obtained with these

Table 4 The affinity of compounds for adrenoceptor subtypes labelled by [³H]-prazosin

Compound	Cloned $\alpha_{1A/D}$ K_i (nM)	Rat cortex + CEC K_i (nM)	Cloned α_{1B} K_i (nM)	Rat liver K_i (nM)
Prazosin	0.32 $\pm$ 0.02	0.18 $\pm$ 0.33	0.19 $\pm$ 0.02	0.31 $\pm$ 0.03
WB 4101	0.67 $\pm$ 0.07	0.57 $\pm$ 0.10	16.4 $\pm$ 2.0	28.1 $\pm$ 3.1
5-Methyl-urapidil	9.8 $\pm$ 2.8	1.93 $\pm$ 0.23	57.2 $\pm$ 12	189 $\pm$ 33
Phentolamine	6.1 $\pm$ 0.9	4.1 $\pm$ 0.8	39.9 $\pm$ 4.3	96.5 $\pm$ 13
Benoxathian	0.50 $\pm$ 0.02	1.3 $\pm$ 0.2	17.2 $\pm$ 4.4	85.0 $\pm$ 14

Affinities were determined from displacement of [³H]-prazosin binding (0.2–0.3 nM) using membranes derived from rat 1 fibroblasts expressing $\alpha_{1A/D}$ - and α_{1B} -subtypes in comparison to rat liver and CEC-treated cortical membranes. Values represent mean $\pm$ s.e.mean of at least 4 separate experiments. Hill slopes (nH) were not significantly different from unity.

Table 5 Comparative affinities of α_1 -adrenoceptor antagonists for cloned α_1 -adrenoceptor subtypes labelled by [¹²⁵I]-HEAT

Compound	K_i (nM)		
	$\alpha_{1A/D}$	α_{1B}	α_{1C}
Prazosin	0.20 $\pm$ 0.01	0.15 $\pm$ 0.01	0.60 $\pm$ 0.07
WB 4101	0.71 $\pm$ 0.08	4.77 $\pm$ 0.05	0.55 $\pm$ 0.02
5-Methyl-urapidil	5.50 $\pm$ 0.4	144.6 $\pm$ 10.3	2.9 $\pm$ 0.4
Phentolamine	34.5 $\pm$ 2.1	88.2 $\pm$ 6.3	17.5 $\pm$ 1.4
Benoxathian	0.60 $\pm$ 0.0	9.60 $\pm$ 0.7	0.18 $\pm$ 0.01
Piperone	40.5 $\pm$ 4.9	12.7 $\pm$ 1.1	18.0 $\pm$ 2.4

Displacement of [¹²⁵I]-HEAT (50–60 pM) from rat 1 fibroblast membranes expressing each of the α_1 -subtypes was determined with at least 10 concentrations of competing drug. Values represent the mean $\pm$ s.e.mean of 3–4 experiments performed in duplicate. Hill slopes (nH) were not significantly different from unity for any compounds examined.

receptors using tissue membranes and in functional studies to assess the relevance of the cloned data in terms of α_1 -subtype distribution and implication for α_1 -adrenoceptor subtype classification. Whilst heterogeneity amongst α_1 -subtypes suggested by binding and functional experiments has been substantiated by molecular studies, the data obtained in the present study are not consistent with the reconciliation of cloning and pharmacological data obtained with the $\alpha_{1A/D}$ -subtype described by Schwinn & Lomasney (1992). We have demonstrated that the high nM affinity of several α_1 antagonists at cloned $\alpha_{1A/D}$ -adrenoceptors is retained at the α_{1A} -subtype in rat brain revealed in the presence of CEC. In contrast, Schwinn & Lomasney (1992) reported that several agents, in particular benoxathian ($pK_i = 5.1$), phentolamine ($pK_i = 7.0$) and 5-methyl-urapidil ($pK_i = 6.5$) possessed much lower affinity for cloned $\alpha_{1A/D}$ -receptors than obtained in our study. The inconsistency of their affinities at cloned $\alpha_{1A/D}$ -adrenoceptors in relation to functional and binding potency in appropriate tissues therefore suggested the existence of another α_{1A} -subtype, yet to be identified by molecular cloning. Our data are not consistent with this proposal. Several issues need to be resolved in the context of our findings. Firstly, the use of CEC to unmask the α_{1A} population in membrane preparations. Secondly, the discrepancy whereby we have obtained high affinity for phentolamine, benoxathian and 5-methyl-urapidil at cloned $\alpha_{1A/D}$ -adrenoceptors. Thirdly, the significance of the affinity at cloned α_1 -subtypes in relation to tissue distribution and subtype classification.

It is well established that receptor inactivation using CEC is dependent upon concentration, time of exposure, species and nature of the tissue preparation (Tian *et al.*, 1990; Perez *et al.*, 1991; Oriowo *et al.*, 1992). Under appropriate conditions the use of CEC to define α_{1B} populations is based on the finding that it is able to inactivate pure populations of α_{1B}

Figure 2 pA_2 determinations for (a) prazosin and (b) WB 4101 against contractions to noradrenaline in epididymal rat vas deferens. Dose-ratio determinations are shown for each antagonist concentration and a line of best fit to the data is shown. Mean pA_2 determinations from each set of experiments are given in the text.

sites, e.g. when the receptors are transiently expressed in COS-7 cells (Schwinn *et al.*, 1990). Additionally, compounds distinguishing between α_{1A} - and α_{1B} -subtypes are better described in terms of a one site fit in the presence of CEC (Han & Minneman, 1991). However, it should be noted that in some tissues, such as rat heart, CEC does not appear to inactivate all low affinity α_{1B} sites (see Han & Minneman, 1991). Additional resistance to CEC in some tissues could

also be explained by the additional presence of α_{1C} -adrenoceptors which have intermediate sensitivity to CEC (Schwinn *et al.*, 1990), although in rat tissues this is unlikely, given that this subtype appears to be absent or present in very low levels on the basis of Northern blot analysis (Schwinn *et al.*, 1990; 1991). In this study, using membrane preparations, we therefore used a CEC protocol inactivating most of the α_{1B} binding sites rat liver membranes before employing an identical protocol to inactivate these sites in rat cortical membranes. Using this protocol with cloned mammalian receptors, CEC caused a reduction of $15 \pm 2\%$, $95 \pm 5\%$ and $85 \pm 10\%$ ($n = 3$) in the maximal number of [3 H]-prazosin sites at α_{1A} -, α_{1B} - and α_{1C} -subtypes respectively. We are confident that alkylation of the majority of α_{1B} -subtypes in rat cortex had occurred because we observed the well documented biphasic displacement curves to 5-methyl-urapidil, WB 4101 and phentolamine in control tissues, whereas in the presence of CEC all compounds examined had monophasic displacement isotherms with Hill slopes close to unity. In rat cortical membranes, the pharmacological profile of CEC-insensitive sites appeared to be indicative of α_{1A} -adrenoceptors since compounds displayed comparable potencies to that obtained with cloned $\alpha_{1A/D}$ -receptors.

What then are the implications of these findings in the context of the currently proposed classification for α_1 -adrenoceptor subtypes? In the present study the data obtained with cloned α_{1B} -adrenoceptors agree very well with previously reported values obtained under similar assay conditions (Perez *et al.*, 1991). It is well established that rat liver contains predominantly the α_{1B} -subtype (Han *et al.*, 1987; Torres-Marquez *et al.*, 1991) and based on CEC sensitivity and pharmacological profile this is consistent with our data. Thus, we obtained a similar binding profile using both cloned α_{1B} and rat liver membranes. Also consistent with previous findings (Schwinn *et al.*, 1991; Schwinn & Lomasney, 1992) we observed selectivity for α_{1C} - over α_{1B} -subtypes using WB 4101 and 5-methyl-urapidil, with this latter compound also showing selectivity for α_{1C} - over $\alpha_{1A/D}$ -adrenoceptors.

The initial difference between the originally cloned α_{1A} -subtype and subsequently cloned α_{1D} -subtype was based on pharmacological differences in a few compounds and sensitivity to CEC. However, these clones probably code for the same receptor in that they differ by only two amino acids and as discussed, the use of CEC is very much a function of tissue, concentration and time. In this respect, with the exception of phentolamine our data obtained with cloned $\alpha_{1A/D}$ -receptors are similar to data previously obtained with [3 H]-prazosin at cloned α_{1D} -receptors (Perez *et al.*, 1991). The affinities we obtained with phentolamine at cloned $\alpha_{1A/D}$ - and rat brain α_{1A} -receptors (K_S of 6.1 and 4.1 nM respectively) were also generally higher than that previously described on the basis of radioligand binding experiments in rat tissues (Johnson & Minneman, 1987; Han & Minneman, 1991). Nevertheless, our binding data to both cloned and rat brain $\alpha_{1A/D}$ -adrenoceptors are consistent with phentolamine having selectivity for α_{1A} - over α_{1B} -subtypes (Hanft & Gross, 1989; Terman *et al.*, 1990). When we employed [125 I]-HEAT as a ligand to label cloned α_1 -subtypes expressed in rat 1 fibroblast using previously described methods (Schwinn & Lomasney, 1992) a range of α_1 antagonists retained a broadly similar profile to that observed with [3 H]-prazosin. However, we obtained some differences in binding affinity, particularly with phentolamine and the selectivity between subtypes was less apparent for some agents. These differences may be a reflection of different experimental protocols since these subtypes were previously expressed transiently in COS-7 cells or stably in Hela cells (Schwinn & Lomasney, 1992), although in our studies benoxathian still retained high affinity for α_1 -subtypes labelled by [125 I]-HEAT. In addition, benoxathian was a potent inhibitor of NA-stimulated inositol polyphosphate production in rat 1 fibroblasts expressing α_{1C} -receptors ($pK_B = 9.5$, unpublished observations) consistent with the binding affinity at this subtype using [3 H]-prazosin in this cell

line. Indeed, benoxathian is a well established high affinity α_1 -adrenoceptor antagonist and has been shown to have nM affinity at α_1 -adrenoceptors in functional and binding experiments using rat aorta, kidney, cortex and vas deferens and guinea-pig liver (Eltze & Boer, 1992; Aboud *et al.*, 1993; Garcia-Sainz & Romero-Avila, 1993) which does not support the low μ M affinity of this compound determined at all cloned α_1 -subtypes described by Schwinn & Lomasney (1992). Since in our study we observed similar affinities for compounds examined at the cloned $\alpha_{1A/D}$ -receptor and CEC-treated rat cortex, this raises the possibility that this subtype is indeed the functionally relevant α_{1A} -subtype typically located on tissues such as rat hippocampus, cerebral cortex and vas deferens.

Functional studies on rat vas deferens originally demonstrated α_{1A} -adrenoceptors to be the predominant subtype (Han *et al.*, 1987) and consistent with other studies (Aboud *et al.*, 1993) contractions of this tissue by NA were unaffected by CEC, indicative of α_{1A} -adrenoceptors. However, radioligand binding studies have revealed high and low affinity sites for [3 H]-prazosin on smooth muscle preparations (Muramatsu *et al.*, 1990a,b) including epididymal rat vas deferens (Ohmura *et al.*, 1992) in which it has been claimed that compounds such as WB 4101 and benoxathian have selectively for the high affinity component but inhibit agonist-induced contractions through a site corresponding to the low affinity component. Other functional evidence also suggests that a phasic, CEC-sensitive component can be demonstrated in response to single electrical pulses (Mallard *et al.*, 1992; Aboud *et al.*, 1993). Furthermore, the presence of α_{1B} -adrenoceptors in rat vas deferens has also been demonstrated using mRNA detection by Northern analysis (Lomasney *et al.*, 1991). Thus, whilst Minneman *et al.* (1988) were able to demonstrate a CEC-sensitive phosphatidylinositol phosphate response (α_{1B} -mediated), the contractile response to exogenous agonist remained CEC-resistant. The affinities obtained in our study with a range of α_1 -adrenoceptor antagonists against NA-induced contractions of rat vas deferens are consistent with other reported values (Han *et al.*, 1987; Eltze & Boer, 1992; Couldwell *et al.*, 1993) which suggest the contractile element of this tissue in response to exogenous NA to be predominantly mediated via α_{1A} -adrenoceptors. Other studies have been able to demonstrate an excellent correlation between the affinity of antagonists against NA-induced contractions of rat vas deferens and the affinity for α_{1A} sites in rat cortex labelled by [3 H]-prazosin (Eltze *et al.*, 1991; Eltze & Boer, 1992). This is in agreement with our findings in relation to the nature of the cloned rat $\alpha_{1A/D}$ -adrenoceptor which we have found to be characteristic of this subtype in cortex and vas deferens, consistent with Northern analysis of the distribution of this receptor using a rat α_{1A} cDNA probe (Lomasney *et al.*, 1991). Interestingly, recent data obtained with guinea-pig liver membranes have demonstrated the presence of an homogeneous population of α_1 -adrenoceptors which possess pharmacological properties consistent with α_{1A} -adrenoceptors, although Northern analysis indicated the presence of $\alpha_{1A/D}$ -adrenoceptors using an $\alpha_{1A/D}$ cDNA probe (Garcia-Sainz & Romero-Avila, 1993).

Whilst rat cortex and vas deferens appear to be pharmacologically characteristic of the cloned rat α_{1A} - or $\alpha_{1A/D}$ -adrenoceptor, functional evidence indicates the presence of α_1 subtypes inconsistent with current classification. The very high affinity exhibited by prazosin in some tissues, and the different sensitivity of tissues to agents such as niguldipine and CEC is suggestive of further heterogeneity (Flavahan & Vanhoutte, 1986; Muramatsu *et al.*, 1990a; Ford *et al.*, 1993). Indeed, the characterization of subtypes controlling contractile responses in vascular preparations such as rat aorta relates poorly to subtypes defined by binding and molecular cloning (Mir & Fozard, 1989). Furthermore, the use of CEC to distinguish between subtypes may not be definitive since a recent report by Porter *et al.* (1993) has described a subtype on rat parotid acinar cells characteristic of an α_{1B} -adreno-

ceptor which appears to be totally insensitive to CEC. It remains to be established whether additional heterogeneity can be demonstrated by further cloning and expression of α_1 -adrenoceptor subtypes.

In summary, this paper has demonstrated different pharmacological properties of three different cloned α_1 -adrenoceptor subtypes which appear to be similar to the properties

of these subtypes identified in appropriate tissues. In particular, functional and binding data indicate that the cloned rat $\alpha_{1A/D}$ is representative of pharmacologically defined α_{1A} -adrenoceptors in rat vas deferens and cerebral cortex. Therefore, at the present time, reference to this rat α_1 -adrenoceptor subtype as α_{1A} appears to be more appropriate than $\alpha_{1A/D}$ or α_{1D} .

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