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PRELIMINARY BIOCHEMICAL CHARACTERIZATION OF TWO ANGIOTENSIN II RECEPTOR SUBTYPES

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Two angiotensin II receptor subtypes (A and B) are described from rat and human tissues. They have been characterised using specific peptidic and non-peptidic ligands with affinities differing by 1000 fold or more. These subtypes are present in adrenal glomerulosa of both species. Human uterus contains only subtype A, whereas both subtypes are found in rat uterus. Vascular smooth muscle cells in culture express only subtype B. Dithiothreitol totally inhibits binding to subtype B, but enhances the affinity to subtype A. There is a good correlation between the affinities of the selected agonists and antagonists for the two subtypes in the various tissues tested which is a usual requirement for receptor classification.

The renin-angiotensin system plays an important role in cardiovascular function and sodium homeostasis despite low (picomolar) circulating concentrations of the active hormones (1). Since effector organs respond to angiotensin I, II and III (AI, AII and AIII) with 2-3 log differences in potency from tissue to tissue, receptor selectivity for the agonists has been suggested (2). The existence of three receptor subtypes has been proposed from physiological studies using antagonists formed by substituting amino acids 4,7 and 8 of AII (3,4,5,6). Similarly, binding studies have detected sites in various tissues including vascular and nonvascular smooth muscle, adrenal cortex, kidney, liver and brain (7), and receptor subtypes have been described (8,9,10,11). The biochemical approach based on the competition of unlabelled ligand for a site labelled with a radioligand allows the determination of affinity constants and receptor concentration useful for receptor classification (12). The direct characterization of other receptors such as the muscarinic, histaminergic, dopaminergic receptors, has already been successfully achieved by the use of specific antagonists with high affinity.

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Abbreviations used - AI, angiotensin I; AII, angiotensin II; AIII, angiotensin III; BSA, bovine serum albumin; PBS, phosphate buffered saline; DTT, dithiothreitol; SMC, cultured vascular smooth muscle cells.

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Until now, nearly all studies characterizing the AII receptor have been performed with peptides of various length. Non-peptidic antagonists have been reported by Furukawa et al (13), and more recently by Wong et al (14). The purpose of this study was to compare the binding of various peptidic and non-peptidic compounds to membranes obtained from various tissues and species in the hope of obtaining more information on AII receptor subtypes.

MATERIALS AND METHODS

Materials - [^{125}I]AII (2200 Ci/mmol) was obtained from Anawa (Wangen, Switzerland). CGP 42112A is a peptide with the formula Nicotinic acid-Tyr-(N^{Σ}-Benzyloxycarbonyl-Arg)Lys-His-Pro-Ile-OH. It has been shown to be an AII antagonist in a rabbit aorta ring assay (unpublished observation). Ex 89 is a non-peptidic antagonist described by E.I. Du Pont de Nemours (European patent application 253310) with the formula 2-butyl-4-chloro-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]-5-(hydroxymethyl)imidazol. The structures of both compounds were confirmed by amino acid analysis and/or by FAB mass spectroscopy and their purity assessed by thin-layer chromatography (TLC) and/or high performance liquid chromatography. All unlabelled octa- and heptapeptides were obtained from Bachem (Bubendorf, Switzerland).

Membrane preparation - Tissues (rat and human adrenal glomerulosa and uterus, and human renal arteries) were homogenized in 20 mM sodium bicarbonate (Polytron, 3 x 8 s) and centrifuged at 600 g for 20 min at 4°C. The pellet was resuspended and treated similarly once more. The pooled supernatants were centrifuged at 30000g for 20 min. The supernatant and the lipid floating layer were discarded and the membrane fraction resuspended in 50 mM Tris-HCl buffer, pH 7.4, containing NaCl (125 mM), $MgCl_2$ (6.5 mM), EDTA (1mM), BSA (2 mg/ml), using a tight fitting teflon-pestle homogenizer. Finally, a cocktail of peptidase inhibitors (antipain, phosphoramidon, leupeptin, pepstatin A, bestatin, amastatin, each at 1.25 µg/ml) was added. Cultured rat aorta smooth muscle cells (SMC) and human renal artery SMC were washed with phosphate buffered saline (PBS) and harvested using a rubber policeman. The cells were homogenized (Polytron, 1 x 8 s) and centrifuged for 30 min at 30000g. The pellet was resuspended as described above. The membrane preparations were held in aliquots at -80°C until used. There was no apparent loss of AII binding activity.

Binding assay - Total reaction volume was 250 µl, consisting of 200 µl membranes (10-35 µg protein), 25 µl [^{125}I]AII (0.15 nM) and 25 µl of varying concentrations of unlabelled competitor. Incubation time was 60 min at 25°C. The reaction was terminated by adding 2 ml of ice-cold PBS. Bound and free radioactivity were separated by immediate filtration through Whatman GF/F filters which were rinsed three times with 2 ml cold PBS. The trapped radioactivity was determined in a γ-counter (Pharmacia-LKB, Uppsala, Sweden) at 80% efficiency. Non-specific binding was measured in the presence of 1 µM unlabelled AII and did not exceed 10% of the total binding which itself was always less than 15% of the added radioactivity. The degradation of the radioligand, as measured by TLC after incubation with membrane, was always less than 15%.

Protein determination - The method of Lowry et al (15) was followed using bovine serum albumin (BSA; Fluka, Buchs, Switzerland) as standard.

Data analysis - Equilibrium dissociation constant (K_d) and concentration of receptor sites (B_{max}) were determined from the competition curves using the iterative curve-fitting program LIGAND (16). The IC_{50}'s were estimated using a four parameter logistic dose-response analysis (17). Assuming a competitive interaction, inhibition constants (K_i) were calculated following the formula $K_i = IC_{50}/(1+L/K_d)$ where L is the concentration of radioactive ligand and K_d its dissociation constant. The data were obtained from experiments using a minimum of 6 concentrations of each competitor.

RESULTS

In rat adrenal glomerulosa, AII inhibited [^{125}I]AII binding with an IC_{50} of 1 nM (Fig.1A). Scatchard analysis suggested a simple interaction with a single population of receptor sites (not shown). In contrast, CGP 42112A and Ex 89 produced a biphasic displacement curve clearly suggestive of receptor heterogeneity. Analysis of the curves indicated the presence of two populations of receptor sites in a ratio of 40/60. Furthermore, it suggested that CGP 42112A bound with a much higher affinity to the 40% population (type 'A'), whereas Ex 89 bound with higher affinity to the 60% population (type 'B'). A similar result was obtained with human adrenal glomerulosa membranes, except that the ratio of the two types was different (Fig.1B). In order to confirm this finding, the experiment was repeated in the presence of 2 x 10^{-6}M Ex 89, a concentration that would block all B receptors (Fig.1C). A further experiment was performed in the presence of 5 x 10^{-7}M CGP 42112A to block the A receptors (Fig.1D). The results quite clearly show

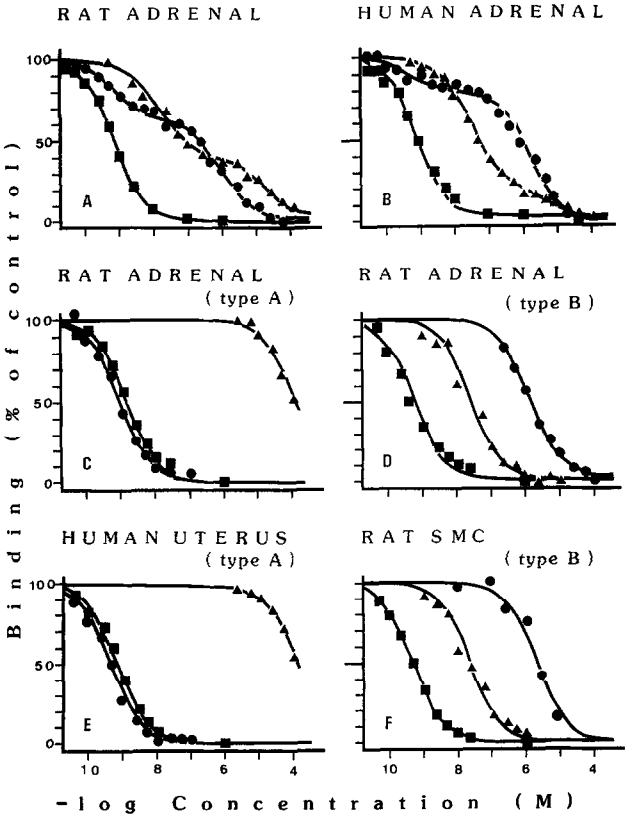

Fig. 1
Competition curves with angiotensin II (■), CGP 42112A (●) and Ex 89 (▲). The panels A,B,E,F represent the binding without any blockade. The results in panel C and D were obtained with specific blockade of type B and A with Ex 89 and CGP 42112A respectively.

that two distinct receptor populations are present in rat adrenal glomerulosa with different specificities for these two compounds, but with a similarly high affinity for angiotensin II itself.

The same blockers were then used to characterize AII binding in other rat and human tissues. In human uterus only type A receptors were found (Fig.1E), whereas in rat aortic smooth muscle cells binding was to type B only (Fig.1F). The K_d and B_{max} values for AII were calculated from the inhibition curves after specific blockade of type A or B (Table 1). In contrast to human, rat uterus was shown to possess both types of receptors, also in a ratio of 40/60. The K_d was similar for each receptor type in the various tissues with a mean of 1 nM (range 0.63 to 1.77) for type A and 0.8 nM (range 0.59 to 1.05) for type B. The B_{max} however varied widely between the different tissues and sometimes also between the individual membrane preparations. In the rat uterus, the sum of both receptors was more than 10 fold less than in rat adrenal and 2-3 fold less than type A in human uterus. Human SMC isolated from renal artery tissue and grown in culture also showed only type B, whereas both types of receptor were found in membranes prepared from the tissue itself (K_dA 0.63 nM, K_dB 1.05 nM; B_{max}A 14.4 fmol/mg, B_{max}B 13.6 fmol/mg), suggesting that the two receptor populations may be present in different cell types.

The effect of dithiothreitol (DTT) was also investigated. This agent inhibited binding of AII to rat aorta SMC (type B) with an IC_{50} of 5 mM, whereas it increased binding to human uterus (type A), with a maximum reached at 2.5 mM (Fig. 2). DTT at 2mM increased the affinity of AII binding

Table 1. DISSOCIATION CONSTANT (K_d) AND MAXIMUM BINDING CAPACITY (B_{max})

	SPECIES	TISSUE	N	K_d (nM)	SEM	B_{max} (fmol/mg protein)	SEM
TYPE A - DTT							
	RAT	UTERUS	3	1.78		119	
		ADRENAL	5	0.86	0.09	885	122
	HUMAN	UTERUS	8	0.66	0.07	928	121
		ADRENAL	4	0.65	0.03	325	23
TYPE A + DTT							
	RAT	UTERUS	5	0.51	0.09	82	13
		ADRENAL	8	0.25	0.06	542	58
	HUMAN	UTERUS	4	0.25	0.03	1029	144
	HUMAN	ADRENAL	3	0.32		384	
TYPE B	RAT	UTERUS	3	0.66		236	
		ADRENAL	5	0.32	0.03	771	102
		SMC	7	0.67	0.09	891	92
	HUMAN	ADRENAL	3	1.07		390	
		SMC	4	0.53	0.02	365	135

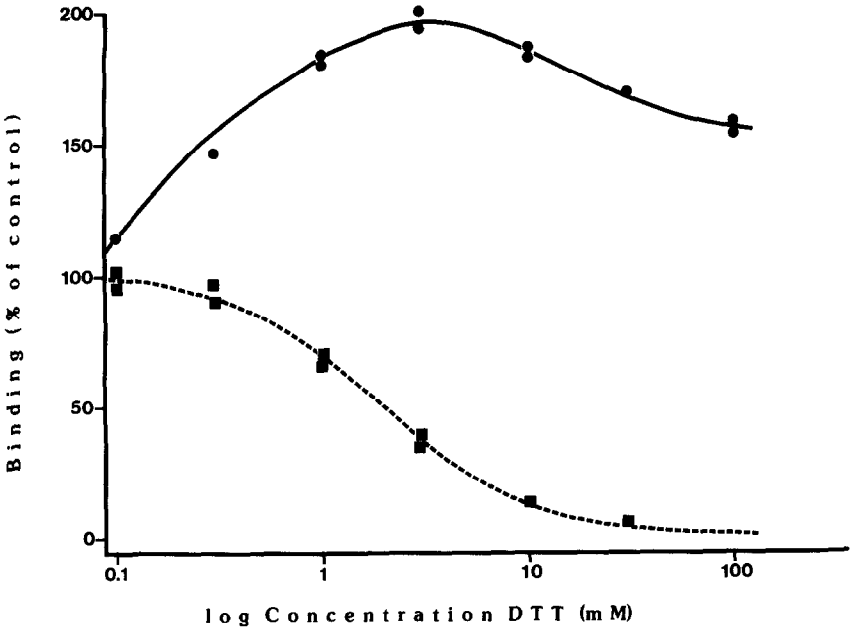

Fig. 2
Effect of various concentrations of dithiothreitol on the binding of 0.15 nM [^{125}I]angiotensin II in human uterus (●) and in rat aorta smooth muscle cells (■).

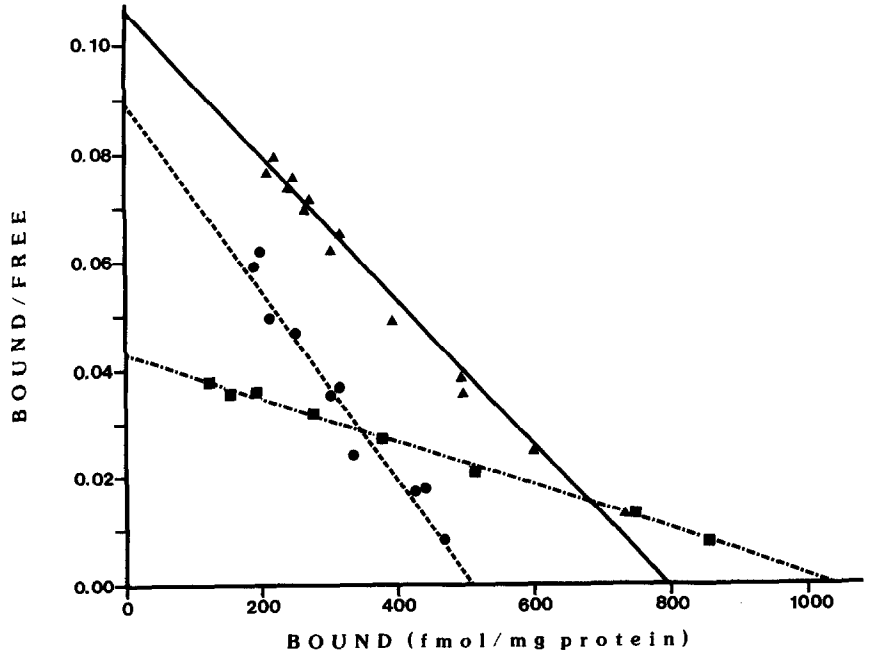

Fig. 3
Effect of 2 mM dithiothreitol on angiotensin II binding in rat adrenal (Scatchard plot).
Type A without (■) and with (●) dithiothreitol.
Type B without (▲) dithiothreitol.

Table 2. INHIBITION CONSTANTS (K_i) IN nM FOR ANGIOTENSIN II AGONISTS AND ANTAGONISTS FOR EACH RECEPTOR TYPE (A AND B)

COMPOUND	TYPE	DTT	Rat Uterus	Human Uterus	Rat Adrenal	Human Adrenal	Rat SMC	Human SMC
AIII	A	-	0.85	0.38	0.77	0.45	nd	nd
	A	+	0.36	0.15	0.17	0.21	nd	nd
	B	-	1.11	nd	0.57	5.49	1.95	0.70
(Sar^1Ile^8)-AII	A	-	0.79	0.45	0.63	0.28	nd	nd
	A	+	0.15	0.14	0.11	0.13	nd	nd
	B	-	0.54	nd	0.39	0.97	0.64	0.43
($desAsp^1Ile^8$)-	A	-	0.92	0.60	0.86	0.48	nd	nd
AII	A	+	0.32	0.17	0.16	0.16	nd	nd
	B	-	2.03	nd	1.41	10.69	2.91	1.59
CGP 42112A	A	-	0.98	0.45	0.73	0.35	nd	nd
	A	+	0.41	0.23	0.28	0.10	nd	nd
	B	-	919	nd	568	3732	1750	741
EX 89	A	-	4960	100000	100000	100000	nd	nd
	A	+	9050	>100000	100000	>100000	nd	nd
	B	-	24.40	nd	16.40	29.7	26.20	27.70

nd = subtype not detectable.

to type A in all tissues tested (Table 1). The B_{max} remained unaffected, except in rat adrenal, where there was a drop of about one half (Fig. 3).

In addition to AII, CGP 42112A and Ex 89, displacement curves for (Sar^1Ile^8)-AII, AIII and ($desAsp^1Ile^8$)-AII were performed. The affinities of these compounds (expressed in K_i) for each of the two types did not differ by more than a factor of 2 (or 10 in human adrenal) between the tissues or species tested (Table 2). None of the hepta- and octapeptides tested gave K_i values for the two receptor types which differed by more than a factor of 10, whereas the corresponding factors for CGP 42112A and Ex 89 were 1000 and >4000 respectively. Similar results (unpublished data) were obtained in solubilized membranes from rat adrenal and human uterus using the method of Capponi (18).

DISCUSSION

The data reported here demonstrate the presence of two receptor subtypes, here termed A and B. They can be differentiated by the use of specific ligands with affinity differences of 1000 fold and more. Subtypes of adrenoreceptors have also been characterised in this way (19). The two AII subtypes appear to be widespread, having been found in various proportions in adrenal, uterus and vascular tissue of both rat and man, although in some tissues, e.g. human uterus, only one subtype is found. The affinity constants of the compounds tested vary little between tissues or species, which is one of the main criteria used for receptor classification

(12). Preliminary data (not shown) suggest that these findings also hold true for tissues from rabbit and marmoset. The finding that primate uterus has only subtype A, whereas in the rat (and rabbit) uterus both subtypes are found is interesting, considering the different uterine morphology in these groups of species.

Subtypes A and B are not equally affected by DTT, an agent able to reduce disulphide bridges. At 10 mM, subtype B is almost totally blocked, whereas at the same concentration binding to subtype A is enhanced (K_d increased, B_{max} decreased). This is an important observation as many workers include DTT in their receptor assays, usually to prevent ligand degradation (20), and compare their results with functional tests performed in the absence of this agent. In our experiments the cocktail of peptidase inhibitors used was able to prevent degradation.

A number of biochemical and physiological studies have indicated the existence of different populations of AII receptors. Peach and Levens (4) claimed that their type I antagonists bound to receptors located in the pressor and myotropic systems, whereas type II antagonists were effective in the adrenal medulla, renal tubules and sympathetic nerve endings, suggesting that the receptors in these groups of tissues were different. Miyazaki et al (10) reported differences between AII receptors in bovine ovary and adrenal cortex, based on the effect of DTT. Capponi et al (21), however, reported that the angiotensin receptors in dog adrenal and uterus had similar physico- chemical properties.

Further work is clearly needed to correlate these AII subtypes with those reported by other groups, and also to identify their functions and post-receptor mechanisms. Pharmacologically, it will be important to see whether the characterization of these subtypes will result in the development of AII antagonists with selective biological properties.

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