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The binding of [^{125}I]-angiotensin to rat renal epithelial cell membranes

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1 Specific high affinity binding sites for [^{125}I]-angiotensin II have been identified in crude basolateral and brush border membranes from rat renal cortex.

2 A central high affinity site, K_D 0.62 nM; B_{max} 299 fmol/mg was identified as part of a complex multicomponent binding system.

3 This high affinity site was saturable and exhibited specificity for angiotensin II analogues and closely related peptides but not for bradykinin, substance P or peptide fragments of angiotensin II.

4 Specific [^{125}I]-angiotensin II binding was partially dependent on NaCl. Absence of NaCl resulted in a decrease in B_{max}, had no effect on the rate of association but increased the rate of dissociation of [^{125}I]-angiotensin from its binding site.

Introduction

The polypeptide angiotensin II has a complex role in renal function. It is well established that angiotensin produces antinatriuretic and antidiuretic effects, but whether these changes are by direct action on epithelial cells as suggested by Munday, Parsons & Poat (1972), Harris & Young (1977) or as a consequence of changes in renal blood flow proposed by Bonjour & Malvin (1969) has not been resolved. More recently it was demonstrated that the stimulation of ion pump activity in renal cortex slices *in vitro* can be blocked by α- but not β-adrenoceptor antagonists and the mediation of noradrenaline in the angiotensin response was suggested (Brunton, Parsons & Poat, 1978). Such an effect could be brought about by presynaptic angiotensin receptors modulating the stimulated release of noradrenaline in a similar fashion to those identified in other peripheral tissues (Starke, 1977).

Angiotensin is produced intrarenally in cells of the juxtaglomerular apparatus and it has been suggested that locally produced angiotensin plays a part in the control of glomerular filtration (Arundell & Johns, 1982).

Thus, from the proposed renal sites of action of angiotensin, binding sites for the polypeptide would be anticipated in renal arterioles, renal tubular epithelial cells and cells from the glomerulus. In the glomerulus there have been a number of reports of such sites, for example high affinity angiotensin binding sites have been identified in glomeruli and glomerular basement membranes (Sraer, Sraer, Ardaillou & Mimoune, 1974; Sraer, Baud, Cosyns, Verroust, Nivez & Ardaillou, 1977) and in glomerular mesangial cells (Foidart, Sraer, Delarue, Mahieu & Ardaillou, 1980). The present study describes and characterizes [^{125}I]-angiotensin binding sites in another potential renal target site, membranes prepared from epithelial cells.

Methods

Preparation of membranes and binding

Male Wistar rats weighing approximately 250 g were killed by cervical dislocation and the kidneys removed immediately. The renal cortex was dissected on ice and homogenized in 10% (w/v) isolation medium containing 250 mM sucrose, 10 mM triethanolamine HCl and 0.1 mM phenylmethylsulphonyl flouride (PMSF). A crude basolateral and brush border membrane fraction was prepared by differential centrifugation as described by Heidrich, Kinne, Kinne-Saffran & Hannig (1972). This fraction was used for ligand binding assays. Membranes (15–25 μg protein) were incubated with [^{125}I]-angiotensin II (specific activity 1880 Ci per mmol) usually at a concentration of 1 nM for 5 min at 22°C in 20 mM Tris-HCl buffer, pH 7.4, containing 120 mM NaCl, 5 mM Na_2EDTA, 0.1 mM PMSF and 0.2%

bovine serum albumin in a final volume of 250 μl. Specific binding was defined by addition of 1 μM unlabelled angiotensin (Ile^5 angiotensin II) to half of the tubes. After incubation, free and bound ligand were separated by dilution with 10 ml ice cold buffer and rapid filtration through Whatman GF/B filters under vacuum. Bound radioactivity was counted in a Beckman Gamma counter at 76% minimum counting efficiency. Filter blanks from identical incubations without membranes were routinely determined.

Chromatography

The extent of angiotensin degradation was determined by thin layer chromatography as described by Goodfriend & Simpson (1981). In some experiments incubations were terminated by the addition of an equal volume of glacial acetic acid and precipitated protein removed by centrifugation. To examine degradation of bound peptide some incubations were terminated by centrifugation. The pellet was then washed and resuspended in 50% glacial acetic acid. Aliquots of incubated samples were co-chromatographed with zero time controls and untreated [^{125}I]-angiotensin II on cellulose plates and developed with 3% NH_4OH; sec-butanol (35:105). Developed dried plates were cut into 0.5 cm strips and counted in a Beckman Gamma counter.

Enzyme assays

The purity of the membrane fraction in binding studies was assessed by assaying alkaline phosphatase (Eicholz & Crane, 1965) and ouabain-sensitive Na^+,K^+-ATPase (Lewis, Elkin, Michell & Coleman, 1975) in various fractions. Enzyme activity is expressed per mg protein, protein being determined using Coomassie brilliant blue dye as described by Bradford (1976).

Materials

Monoiodinated [^{125}I]-angiotensin II (sp. act. 1880 Ci/mmol) was obtained from N.E.N., Boston. Angiotensin and its analogues were obtained from Cambridge Research Biochemicals Ltd.

Results

The composition of the membrane preparation used was examined by the use of specific marker enzymes, alkaline phosphatase being a marker for brush border membranes and ouabain-sensitive Na^+,K^+-ATPase found predominantly on basolateral membranes. The specific activities of these two enzymes and specific [^{125}I]-angiotensin binding in various fractions produced during the preparation are shown in Table 1. Alkaline phosphatase activity was enriched some 14 fold in the final pellet compared with the washed starting homogenate, and Na^+,K^+-ATPase activity increased six fold. Specific angiotensin binding (expressed per mg protein) was also highest in this fraction. These results suggest that the fraction (P5) consists predominantly of crude basolateral/brush border membranes.

Table 1 Enrichment of specific activities for alkaline phosphatase and Na^+K^+ATPase and specific [^{125}I]-angiotensin II binding in different fractions from the preparation of kidney cortex crude basolateral and brush border membranes

Fraction	*Specific activity* (μmol mg^{-1} min^{-1})	*Enrichment*	*Total activity* (μmol min^{-1} mg^{-1})	*Recovery* (%)
Alkaline phosphatase				
S2	0.419 ± 0.05	1.00	8.35 ± 0.34	100.0
SP3	0.767 ± 0.03	1.83	5.01 ± 0.54	60.0
P4	3.406 ± 0.05	8.13	1.01 ± 0.02	12.1
P5	5.683 ± 0.49	13.56	0.19 ± 0.02	2.3
Ouabain-sensitive Na^+K^+-ATPase				
S2	0.021 ± 0.01	1.00	89.68 ± 7.68	100.00
SP3	0.027 ± 0.003	1.32	26.02 ± 3.54	29.0
P4	0.074 ± 0.001	3.69	8.39 ± 0.02	9.4
P5	0.126 ± 0.015	6.23	0.45 ± 0.05	0.5
Specific [^{125}I]-angiotensin II binding (fmol/mg)				
S2	220.6 ± 75.5			
SP3	99.4 ± 34.1			
P4	117.1 ± 49.3			
P5	437.3 ± 61.1			

This observation was confirmed upon electron microscopic examination of the final P5 pellet. The micrographs showed a predominantly vesicular profile, typical of plasma membranes, mitochondrial and ribosomal contamination being at a minimum.

Specific binding of [^{125}I]-angiotensin II to this membrane preparation was saturable over the concentration range (0.7–1 nM) and analysis of this data gave a K_d of 0.62 nM and B_{max} of 299 fmol per mg protein. When tested over a greater concentration range (0.1–5 nM) the specific binding was seen to be of a multicomponent nature as shown in Figure 1. A binding site with lowest affinity, saturating at approximately 5 nM and a binding site with higher affinity were also observed in the concentration range tested. The rest of the study concentrated upon the central high affinity site with a K_d of 0.62 nM since the circulating concentration of angiotensin is between 0.05 and 0.6 nM (Powell-Jackson & MacGregor, 1976). Thus, this site may well be operative under physiological conditions.

The affinity constant for [^{125}I]-angiotensin specific binding was estimated by a second method, from association and dissociation constants (Figure 2). Fitting of initial rate binding data to pseudo first order and second order rate equations gave an association rate constant of $11.8 \times 10^6\,M^{-1}s^{-1}$ and a dissociation rate of $6.5 \times 10^{-3}\,s^{-1}$ and thus an affinity constant of 0.55 nM, this value being in good agreement with that produced by saturation analysis.

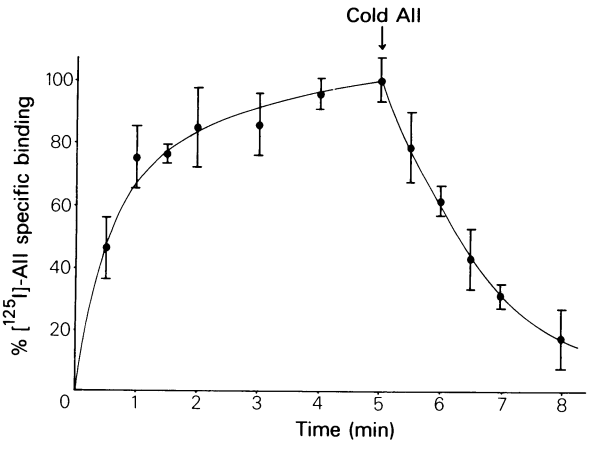

Figure 2 Association and dissociation of [^{125}I]-angiotensin II specific binding to rat renal cortex basolateral and brush border membranes. The rate of association of specific [^{125}I]-angiotensin binding with renal cortex membranes was assessed by increasing the incubation period from 0.5 to 5 min at 22°C. After 5 min incubation, 1 μM unlabelled angiotensin II was added and the rate of dissociation measured by diluting and filtering the tube contents at specific time intervals after this addition. Each point represents the mean of four determinations; vertical lines are s.e.mean.

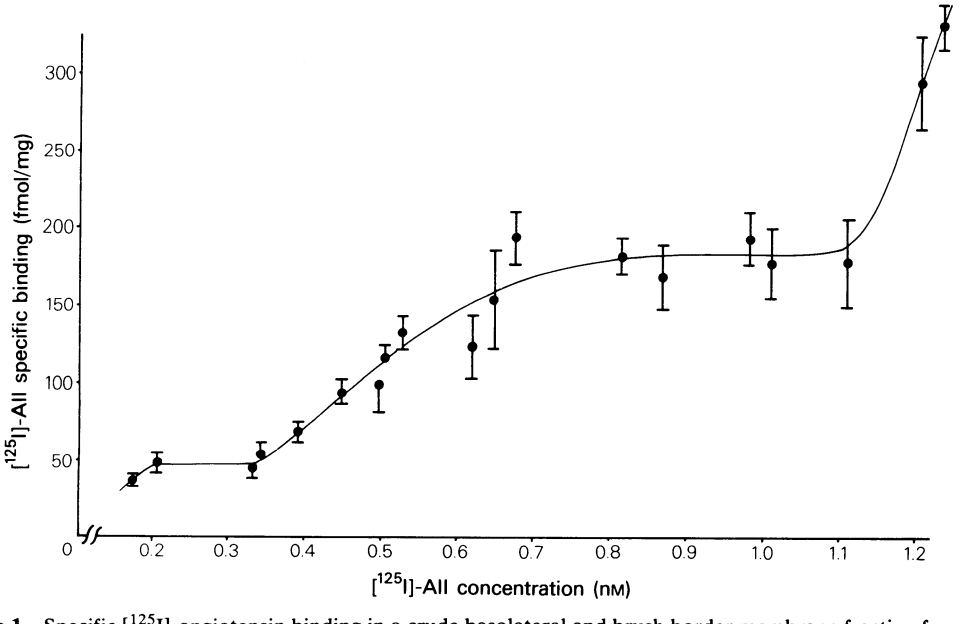

Figure 1 Specific [^{125}I]-angiotensin binding in a crude basolateral and brush border membrane fraction from rat kidney cortex. [^{125}I]-angiotensin binding (0.2–1.2 nM) was determined as described in the methods using 1 μM unlabelled angiotensin II to define specific binding. Saturation analysis of the central high affinity site gave K_D 0.62 nM and B_{max} 299 fmol/mg. Each point is the mean of four determinations; vertical lines are s.e.mean.

The effect of some angiotensin II analogues and fragments upon specific [^{125}I]-angiotensin binding to crude epithelial cell membranes is shown in Table 2. The results are expressed as IC_{50} and K_i values, and demonstrate the specificity of the binding site for angiotensin II analogues and components of the renin-angiotensin system. Angiotensin II fragments and unrelated peptides such as substance P do not displace [^{125}I]-angiotensin binding. Of the angiotensin analogues the competitive antagonists [Sar^{1}Leu^8]-AII and [Sar^{1}Ile^8]-AII were the most potent with the angiotensin III competitive antagonist [Des Asp^1 Ile^8]-AII and [Sar^1 Ala^8]-AII being only a little less potent than [Ile^5]-AII. Components of the renin-angiotensin system were relatively weak in displacing specific [^{125}I]-angiotensin II binding, angiotensin I and angiotensin III having 10% and 6.8% of the angiotensin II displacing activity and renin substrate being 40 times less potent than the native hormone. It is of particular interest that two dipeptides, L Asp-L Arg, L Ile-L His were without activity in displacing angiotensin II specific binding since the polypeptide is known to be particularly susceptible to enzymatic degradation and epithelial cells have a high content of protease and other degradative enzymes. Examination of angiotensin breakdown during the experiments by thin layer chromatography demonstrated that 50% of the angiotensin remained in its native form after 5 min incubation at 22°C, even though the buffer normally contained 0.1 mM PMSF and 5 mM Na_2 EDTA. This degradation was substantially inhibited by the addition of 5 mM DTT to the incubation buffer (approximately 85% of angiotensin now remaining after 5 min at 22°C), however the presence of DTT substantially inhibited specific [^{125}I]-angiotensin binding to the membranes and its use was abandoned. Degradation of the peptide was not affected by the addition of 100 μM bacitracin or 1 mM PMSF. However, following release of bound radioactivity from the membranes only one peak of radioactivity corresponding to native angiotensin was observed after t.l.c. (R_F of [^{125}I]-angiotensin, 0.3; R_F of [^{125}I]-AII bound, 0.29).

The effect of sodium on specific [^{125}I]-angiotensin binding

In other preparations specific [^{125}I]-angiotensin binding is affected by the presence of sodium ions. Binding is increased in the presence of sodium in cerebellar cortex membranes (Bennett & Snyder, 1980), adrenal cortex (Glossmann, Baukal & Catt, 1974a) and vascular tissue (Gunther, Gimborne & Alexander, 1980). In renal cortical epithelial cell membranes specific [^{125}I]-angiotensin binding was also sodium-dependent as shown in Figure 3. In the absence of sodium, specific [^{125}I]-angiotension binding was 49.2 fmol per mg protein and increased to a maximum of 210.0 fmol per mg in control buffer containing 120 mM NaCl. Addition of sucrose at 120 mM had no effect upon the specific [^{125}I]-angiotensin binding. At concentrations greater than 120 mM sodium, specific binding was decreased, presumably due to a charge effect. High sodium concentrations had a similar effect on [^{3}H]-angiotensin binding in adrenal glomerulosa cells as reported by Gurchinoff, Khairallah, Devynck & Meyer (1976).

The cation specificity of the effect was investigated

Table 2 Displacement of [^{125}I]-angiotensin II specific binding to crude basolateral and brush border membranes of kidney cortex by angiotensin II, analogues and unrelated peptides

Displacer	IC_{50} (nM)	K_i (nM)
[Sar^1-Leu^8]-AII	12.5	3.78
[Sar^1-Ile^8]-AII	27.0	9.26
[Ile^5]-AII	58.0	20.28
[Des Asp^1-Ile^8]-AII	100.0	31.44
[Sar^1-Ala^8]-AII	150.0	49.60
[Ile^5]-AI	580.0	180.00
[Ile^4]-AIII	850.0	279.58
Renin substrate	2340.0	752.14
Bradykinin	1.0 mM	0.32 mM
Converting enzyme inhibitor	1.0 mM	0.41 mM
Substance P	> 1.0 mM	
L-Asp-L-Arg	> 1.0 mM	
L-Ile-L-His	> 1.0 mM	

[^{125}I]-angiotensin II (1 nM) was incubated for 5 min at 22°C with 15–25 μg protein and various concentrations of different peptides. From resulting displacement curves IC_{50} values, i.e. the concentration of displacer producing 50% reduction in specific binding, were obtained. K_i values were calculated using the Cheng-Prussof equation.

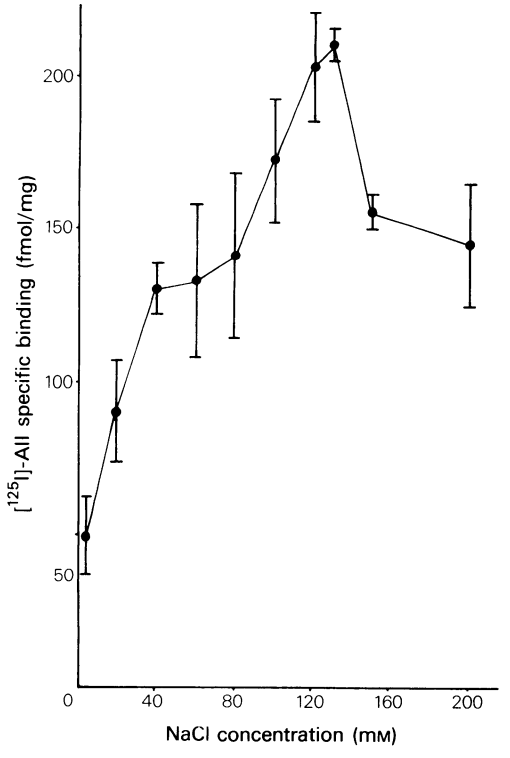

Figure 3 Effect of sodium chloride concentration on specific [125I]-angiotensin II binding to kidney cortex basolateral and brush border membranes. Kidney cortex membranes were incubated in 20 mM Tris-HCl buffer, pH 7.4, containing 5 mM Na_2 EDTA, 0.1 mM PMSF, 0.2% BSA and various concentrations of NaCl for 5 min at 22°C. Binding of [125I]-angiotensin II was estimated with 1 nM ligand and 1 μM angiotensin II to define specific binding. Each point represents the mean of four determinations; vertical lines are s.e.mean.

by replacing 120 mM NaCl by 120 mM KCl, 120 mM LiCl, 120 mM RbCl or 120 mM choline chloride. Of these ions only choline chloride was unable to maintain the stimulated rate of specific binding (55 fmol per mg in the presence of choline chloride as compared with 49 fmol per mg in the absence of NaCl). Replacement of sodium by potassium, lithium or rubidium produced levels of [125I]-angiotensin binding similar to controls in the presence of NaCl. Similarly, replacement of 120 mM NaCl with 120 mM NaH_2PO_4 NaSCN, NaBr or 60 mM Na_2SO_4 produced similar levels of specific [125I]-angiotensin binding as controls. Thus, the binding appears to be cation rather than anion dependent.

The effects of sodium upon the association and dissociation constants were examined and the results are shown in Figure 4. It can be seen that sodium has

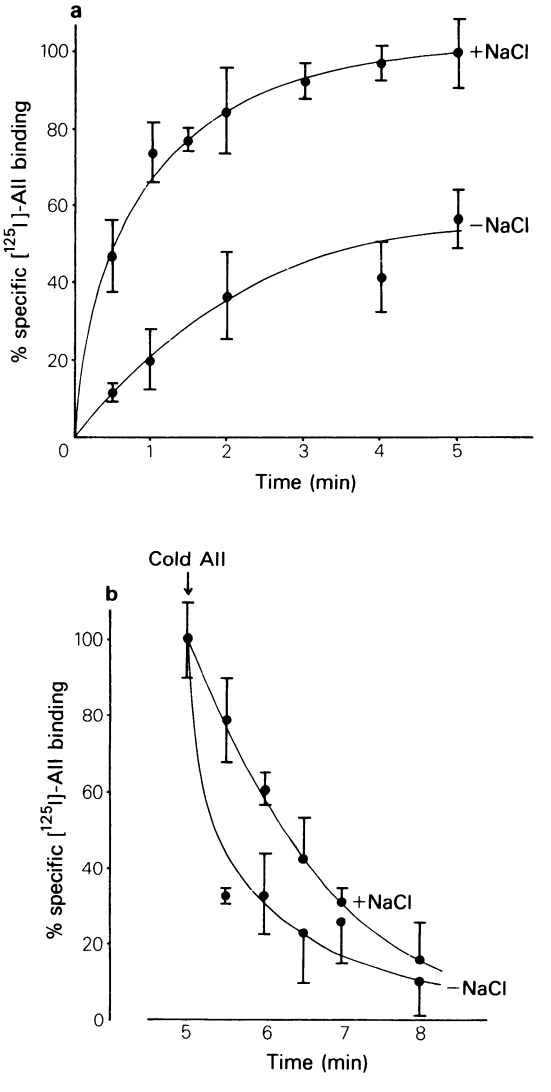

Figure 4 The rates of association and dissociation of specific [125I]-angiotensin II binding in the presence and absence of NaCl. (a) The rate of association of specific [125I]-angiotensin II binding with renal cortex crude basolateral and brush border membranes was assessed by incubating 1 nM [125I]-angiotensin II with membrane protein (and 1 μM cold angiotensin II in half of the tubes) for various time intervals between 0.5–5 min at 22°C, in the presence or absence of 120 mM NaCl. (b) After 5 min incubation in buffer with or without 120 mM NaCl at 22°C, 1 μM unlabelled angiotensin II was added and the rate of dissociation of [125I]-angiotensin II assessed by dilution and filtration at specific time intervals after this addition. Each point represents the mean of four determinations; vertical lines are s.e.mean.

little influence upon the rate of association of [^{125}I]-angiotensin II but markedly alters the rate of dissociation ($6.5 \times 10^{-3}\,s^{-1}$ in the presence of sodium compared with $27.0 \times 10^{-3}\,s^{-1}$ in the absence of sodium). Thus, the overall effect of Na lack upon [^{125}I]-angiotensin binding is to reduce the maximal binding capacity, to have little significant effect upon the affinity constant but to increase the rate of dissociation of [^{125}I]-angiotensin from its binding site.

Discussion

Specific [^{125}I]-angiotensin binding sites have been identified in membranes from rat renal cortex. The enzymatic profile and electron microscopic examination suggests that the fraction is essentially a mixture of brush border and basolateral membranes. This is consistent with the view of Heidrich *et al.* (1972), who first used this preparation to produce transporting vesicles of brush border and basolateral membranes for ion transport studies. These workers extensively characterized the preparation and produced electron micrographs showing similar structures to those observed in this present study. The binding of the peptide to this preparation occurs in a specific saturable fashion, the different binding components being observed over a relatively narrow concentration range (0.1–5.0 nM). A similar multicomponent binding profile for angiotensin has been reported by Sraer *et al.* (1977) in renal glomerular basement membranes. The binding component with a K_d of 0.62 nM and a maximal binding capacity of 299 fmol per mg protein was chosen for further investigation since this falls within the circulating concentrations of the hormone of 0.05–0.6 nM as reported by Powell-Jackson & McGregor (1976) and thus this binding site may be of physiological importance. This binding site showed a great degree of specificity in that, of the compounds tested, only components of the renin-angiotensin system and angiotensin II analogues would displace the binding. Analogues substituted for phenylalanine in the 8 position are usually competitive antagonists of many angiotensin responses (Regoli, 1979) and such analogues were as potent as the native hormone in competing for the binding. This is consistent with their known mode of action in other systems. The members of the renin angiotensin system also competed for the binding, although with a much reduced potency compared with the native hormone; thus angiotensin I was nine times less potent, angiotensin III 14 times, and renin substrate 40 times less potent. This order of potency is similar to that described in other studies of angiotensin binding (Brown, Douglas & Krontins-Litowitz, 1980; Gunther *et al.*, 1980). Other polypeptides such as substance P and bradykinin were without activity in the binding assay as were two dipeptide fragments Asp-Arg and Ile-His of the angiotensin molecule. This specificity of the binding site not only resembles the known actions of the compounds in physiological responses and upon other binding systems but also lends support to the suggestion that angiotensin degradation is not of major importance in the present study. Although significant loss of the native peptide from the incubation medium was recorded and was not prevented by the inclusion of protease inhibitors, protective proteins and reducing time and temperature of incubation, it is likely that the site will bind only the native hormone. Thus, chromatography of the bound hormone demonstrated only one peak of activity and this co-chromatographed with [^{125}I]-angiotensin II.

This study suggests that epithelial cell membranes do possess specific, saturable binding sites for angiotensin II, similar to those recorded in other target tissues such as adrenal glomerulosa (Glossmann, Baukal & Catt, 1974b), cerebral cortex (Bennett & Snyder, 1980) and mesenteric artery (Gunther *et al.*, 1980). It also suggests that a direct action of the hormone upon epithelial transport as proposed by Munday *et al.* (1972) and Harris & Young (1977) is at least part of the mechanism by which antinatriuresis and antidiuresis is affected. However, the results do not allow any definite statement as to the exact location of these binding sites, since the preparation is enriched in both basolateral and brush border membranes as analysed by the enzymatic profile. This does not preclude the possibility of binding to prejunctional complexes, although these were not seen in electron micrographs of the preparation. The latter possibility has been suggested by Brunton *et al.* (1976) who showed that the *in vitro* actions of angiotensin could be inhibited by α-adrenoceptor antagonists and indeed presynaptic angiotensin receptors which modulate the release of noradrenaline have been observed in many peripheral tissues (Starke, 1977).

One characteristic of angiotensin receptors as identified in other preparations has been that sodium modulates specific binding (Glossmann *et al.*, 1974a; Bennett & Snyder, 1980). This has been tested using membranes from renal cortex epithelial cells and a partial dependence of [^{125}I]-angiotensin II binding upon the presence of sodium was observed. In the absence of sodium binding was not completely abolished, some 25% of the specific binding remaining. The binding was dependent on the concentration of sodium, the half maximal concentration of sodium being about 60 mM. The specificity of the cation did not appear to be very high in that potassium, rubidium and lithium were all equally effective in substituting for sodium without significant loss of binding. The maximal binding capacity was significantly reduced in the absence of sodium, the affinity

constant remaining unchanged. These findings are similar to the effects of sodium on angiotensin binding in certain other target tissues and also with [^{3}H]-sulpiride binding in rat striatal membranes (Freedman & Woodruff, 1981). However, they contrast with a recent report by Wright, Alexander, Ekstein & Gimborne (1982) where the sodium stimulation of specific [^{125}I]-angiotensin binding to rat mesenteric artery membranes was highly specific, potassium having little effect and lithium being inhibitory. Closer examination of our binding data revealed that the dissociation rate constant was significantly increased but the association rate constant was not affected by sodium. This implies that there has been some change in receptor configuration. Again this observation is not without precedent and Theodorou, Hall, Jenner & Marsden (1980) noted changes in affinity constant with sodium upon CNS [^{3}H]-sulpiride binding.

In conclusion, specific saturable binding sites for the hormone angiotensin have been identified in rat renal cortex epithelial cells. These resemble binding sites found in other angiotensin-sensitive tissues such as adrenal cortex (Glossmann *et al.*, 1974b) and cerebral cortex (Bennett & Snyder, 1980). Specific binding is sensitive to sodium and in the absence of sodium the maximal binding capacity and the rate of dissociation are significantly altered, indicating that sodium increases the number of binding sites and alters the configuration of the receptor site. The presence of angiotensin binding sites in renal epithelial cells is consistent with the reported effects of the hormone on renal sodium transport.

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