*Biochem. J.* (1984) **223**, 659–671
*Printed in Great Britain*



# Identification of the angiotensin II receptor in rat mesenteric artery

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(*Received 16 April 1984/Accepted 16 July 1984*)

Specific binding sites of high affinity and low capacity for $^{125}$I-angiotensin II have been identified in a membrane fraction derived from arterial arcades of the rat mesentery. Heterogeneity of binding sites and extensive tracer degradation necessitated the use of nonlinear regression methods for the analysis of radioligand binding data. Forward and reverse rate constants for the high affinity sites obtained by three experimental approaches were in good agreement and gave a dissociation equilibrium constant ($K_d$) of 19–74 pM (95% confidence interval). Affinities for a number of angiotensin-related peptides calculated from competitive binding curves were in the order $^{125}$I-angiotensin II = angiotensin II > angiotensin III > [Sar$^1$,Ile$^8$]angiotensin II > [Sar$^1$,Gly$^8$]angiotensin II. Angiotensin I and biochemically unrelated peptides had virtually no effect on binding of tracer angiotensin II. The divalent cations $Mn^{2+}$, $Mg^{2+}$ and $Ca^{2+}$ stimulated $^{125}$I-angiotensin II binding at concentrations of 2–10 mM, as did $Na^+$ at 50–100 mM. In the presence of $Na^+$ or $Li^+$, $K^+$ had a biphasic effect. The chelating agents EDTA and EGTA were inhibitory, as were the thiol reagents dithiothreitol and cysteine. This study defined angiotensin II binding sites in a vascular target tissue of sufficiently high affinity to interact rapidly with plasma angiotensin II at physiological concentrations.

Chronic elevation or suppression of plasma angiotensin II concentration causes a reciprocal change in pressor responsiveness to the hormone (Kaplan & Silah, 1964; Reid & Laragh, 1965; Dawson-Hughes *et al.*, 1981). Such alterations in vascular sensitivity have been attributed to prior occupancy of the angiotensin receptors by endogenous angiotensin (Thurston & Laragh, 1975; Cowley & Lohmeier, 1977; Oliver & Cannon, 1978) or to changes in the receptors themselves (Brunner *et al.*, 1972) as in other hormone–receptor systems (Baxter & Funder, 1979; Catt *et al.*, 1979). Regulation of the zona glomerulosa angiotensin receptors has been demonstrated and related to altered steroidogenic response to angiotensin (Douglas *et al.*, 1978; Aguilera *et al.*, 1978, 1980). However, most studies to date on the vascular receptors for angiotensin have utilized model tissues such as aorta (LeMorvan & Palaic, 1975; Devynck & Meyer, 1976) or uterus (Rouzaire-Dubois *et al.*, 1975; Devynck *et al.*, 1976) due to the inaccessibility of the vascular target tissues. Consistent with the lack of physiological action of angiotensin in these organs, the affinity of the binding sites for angiotensin II are low in relation to normal plasma concentrations of angiotensin II (10–100 pM), with reported $K_d$ values of 10–50 nM in aorta and 2–10 nM in uterus.

More recently, Gunther *et al.* (1980) and Aguilera & Catt (1981) have used a membrane fraction derived from rat mesenteric arterial arcades, small muscular vessels representative of the resistance vasculature and which are highly sensitive to angiotensin II (Bohr & Uchida, 1967). While developing an assay for the angiotensin receptor in this tissue, we have encountered two obstacles not reported by these authors: heterogeneity of binding sites and extensive degradation of tracer angiotensin II that could not be inhibited without adversely affecting binding capacity. Direct curve fitting methods allowed identification of two classes of saturable binding sites for $^{125}$I-angiotensin II, one having a considerably higher affinity than has been previously observed in this tissue or in any other type of smooth muscle. The high affinity of this class of binding sites is consistent with a function of mediating the vascular response to angiotensin.

Abbreviations used: Sar, sarcosine, *N*-methylglycine; Tos-Phe-$CH_2Cl$, 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one ('TPCK').

## Materials and methods

Tyrosyl $^{125}$I-[Ile$^5$]angiotensin II ($^{125}$I-angiotensin II) was obtained from New England Nuclear. Specific radioactivity (measured by radioimmunoassay) and radiochemical purity (determined by paper chromatography) were within the manufacturers specification (1800Ci/mmol and >98% respectively). Unlabelled angiotensins and other peptides were obtained from Peninsula Laboratories and Cambridge Biochemicals. Glucagon, soya bean trypsin inhibitor, bovine albumin (fraction V, angiotensinase-free), Tos-Phe-$CH_2Cl$, phenylmethanesulphonyl fluoride and neomycin sulphate were obtained from Sigma. Trasylol and di-isopropyl phosphite were obtained from Bayer Pharmaceuticals and Koch-Light Laboratories respectively. All other chemicals were of analytical reagent quality from BDH.

Male Sprague–Dawley rats (325–375g) (Olac Farms), maintained on a normal laboratory animal diet, were used for all experiments. The membrane fraction used for binding studies was prepared by a modification of the method of Wei *et al.* (1976). Rats were killed by a blow to the head, the intestines were immediately removed and transferred to normal saline, and the mesenteric arterial arcades were excised by blunt dissection. Most of the fat and all venous tissue was removed at this stage. The arteries were cleaned of residual adherent fat in 0.25M-sucrose with a 30mm diameter smooth pestle [0.01in. (0.25mm) clearance] Teflon/glass homogenizer (three to five strokes, 6000rev./min low-torque motor). The tissue was then homogenized in 25–50ml of 0.25M-sucrose with a Polytron PT20S (Kinematica GmbH, Switzerland), setting 8 for 2 × 10s. Care was taken to prevent heating of the tissue during homogenization as this greatly reduced the binding capacity of the membrane material. The homogenate was immediately centrifuged at 2000*g* for 10min, the supernatant was decanted and recentrifuged as before. The membrane fraction was then obtained by centrifuging the second supernatant at 100000*g* for 30min. The pellet (approx. 450$\mu$g of protein/arterial arcade) was resuspended by using a small Teflon/glass homogenizer in 50mM-Tris/120mM-NaCl/3.6mM-KCl/1.8mM-$MgCl_2$/4.8mM-$CaCl_2$, pH7.35 at 22°C (Tris/NaCl). All steps were carried out at 0–2°C.

For binding studies, the incubation mixture comprised 100$\mu$l of freshly prepared membrane fraction suspension (20–100$\mu$g of protein) to which was added 100$\mu$l of Tris/NaCl containing 1% (w/v) bovine serum albumin (Tris/albumin) and 2–2000pg of $^{125}$I-angiotensin II. Where additional compounds were added (metal salts, proteinase inhibitors or competing peptides) tracer and additional compound(s) were each added in 50$\mu$l of Tris/albumin. For higher tracer concentrations, the specific activity was reduced by addition of unlabelled angiotensin II and the new concentration verified by radioimmunoassay. Non-specific binding was defined as radioactivity not displaceable by 10$\mu$M unlabelled angiotensin II (added with tracer). All experiments were carried out at 22°C unless otherwise specified. Where the effects of mono- or divalent cations and proteinase inhibitors were investigated, short incubations (15–20min) were used to minimize the effect of changes in rate of tracer degradation on binding. Specific binding is expressed as means ± 1S.D.

Separation of bound and free tracer was achieved by diluting the reaction mixture in 6.5ml of ice-cold Tris/NaCl containing 0.1% bovine serum albumin (filtration buffer) and immediately filtering through 0.2$\mu$m filters (Whatman) prewetted with Tris/albumin. Filters were rinsed with a second 6.5ml of filtration buffer, dried and counted for radioactivity in a Nuclear Enterprises 1612 gamma counter (>90% efficiency). Where the filtrate immunoassay was used (see below), the filtration buffer also contained 0.2% (w/v) neomycin sulphate.

Tracer degradation was assessed by descending paper chromatography on Whatman No. 2 paper and butan-2-ol/3% aq. $NH_3$ (30:11, v/v) as mobile phase. Chromatograms were run for 45h at room temperature, dried and cut into 1cm strips for counting. For quantitative estimation of tracer degradation in binding assays, a sample of each filtrate was incubated for 24h at 4°C with an angiotensin II-specific antiserum. Bound and free radioactivity were separated by using dextran-coated charcoal and values of bound were corrected for the non-specific binding determined in parallel incubations without antiserum. From a standard curve constructed with pure $^{125}$I-angiotensin II the expected total radioactivity was calculated and the fraction of tracer remaining taken as total (expected)/total (measured). Where angiotensin-related peptides were used in displacement experiments, parallel incubations were carried out without $^{125}$I-angiotensin II and degradation of the peptides was determined by radioimmunoassay.

Preparation of antisera and the radioimmunoassay for angiotensin peptides were performed as described by Morton *et al.* (1976), except that angiotensin analogues were iodinated by the solid-phase method of Fraker & Speck (1978), yielding specific radioactivities of 200–300Ci/mmol. Angiotensin II and des-Asp$^1$-angiotensin II (angiotensin III) were assayed by using the same antiserum, which did not discriminate between these peptides. Tyrosyl $^{125}$I-angiotensin I was obtained from New England Nuclear. Antisera were used at final dilutions ranging from 1:4000 to 1:22500.

For chromatographic analysis of receptor-bound radioactivity, the reaction mixture was diluted after incubation under standard conditions (60 min at 22°C) in 5 ml of ice-cold Tris/albumin and centrifuged at 2000*g* for 10 min. The supernatant was discarded and the pellet washed as before. The pellet was then resuspended in 200 µl of Tris/NaCl, transferred to a 100°C water bath for 5 min, centrifuged as above and the supernatant was chromatographed.

Protein was measured by the Lowry method with bovine serum albumin as standard. The statistical analysis of binding data is described in the Appendix.

## Results

Specific binding of $^{125}$I-angiotensin II to the membrane fraction was nonlinear with membrane protein concentration due to the presence of angiotensinase activity (Fig. 1). Non-specific binding was linear with protein concentration but comprised mainly radioactivity absorbed to the filters during separation. In all experiments, net binding amounted to less than 5% of total radioactivity.

Tracer degradation was identified by chromatographic analysis of free radioactivity after incubation under standard conditions (Fig. 2*b*). The predominant tracer metabolites were fragments not containing the *C*-terminal pentapeptide of angiotensin II and consequently were not expected to bind to the angiotensin receptor (Bumpus *et al.*, 1961) or to the antiserum used for angiotensin II immunoassay, since both required this sequence for binding. This was confirmed by direct binding studies with the angiotensin II antiserum (see below) and by chromatographic analysis of eluted receptor-bound radioactivity (Fig. 2*c*). The *C*-terminal heptapeptide (des-Asp$^1$-angiotensin II, angiotensin III) was present as a component of the free radioactivity but proportionately less was observed in the radioactivity eluted from the membrane. Similar results were obtained when these experiments were repeated with a higher initial tracer concentration (20 nM), although the heptapeptide then comprised a larger proportion of the eluted radioactivity (Fig. 2*d*).

A number of proteinase inhibitors were examined for their effect on both binding capacity and tracer angiotensin II degradation (Table 1). No inhibitor or combination of inhibitors effectively prevented degradation of tracer without also reducing binding capacity. Tracer degradation was therefore measured by filtrate immunoassay and the binding data were corrected mathematically.

The accuracy of the filtrate immunoassay was determined by allowing a quantity of $^{125}$I-angiotensin II to be completely metabolized by the membrane fraction preparation (2 h incubation at 37°C with 460 µg of membrane protein/ml). The angiotensinase enzymes were then inactivated by heating at 100°C for 5 min and precipitated protein was removed by centrifugation. Aliquots of the resulting metabolite preparation were then mixed with pure $^{125}$I-angiotensin II such that the added $^{125}$I-angiotensin II represented 0, 20, 50, 75 or 100% of total radioactivity. Samples of each preparation were subjected to immunoassay or chromatographed and the proportion of radioactivity present as $^{125}$I-angiotensin II was determined by calculation of peak areas. The results are presented in Table 2.

Stability of the receptor preparation was assessed by preincubating the membrane fraction

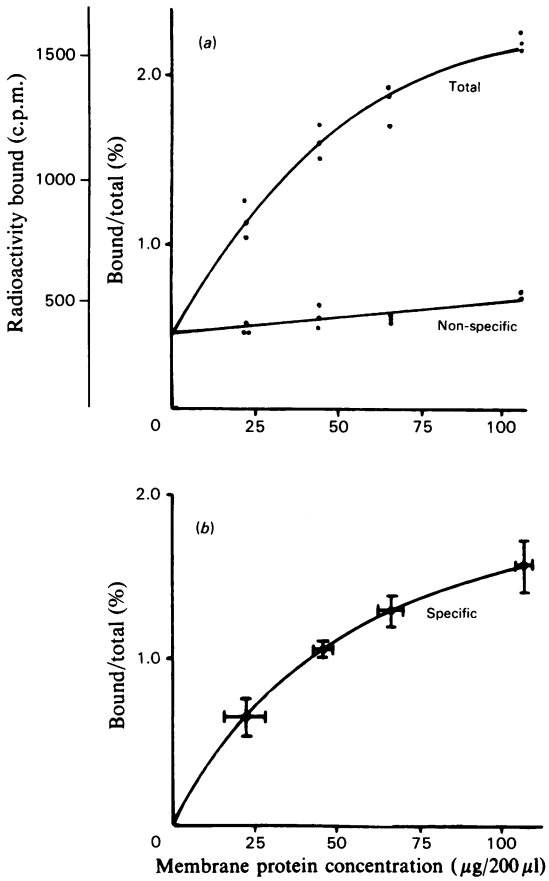


Fig. 1. *Binding of $^{125}$I-angiotensin II to the membrane fraction material as a function of membrane protein concentration*

Incubation was for 60 min at 22°C, initial tracer concentration was 0.3 nM. (*a*) Points denote individual determinations of total and non-specific binding; (*b*) specific binding derived from data in (*a*), points denote mean ± 1 S.D.

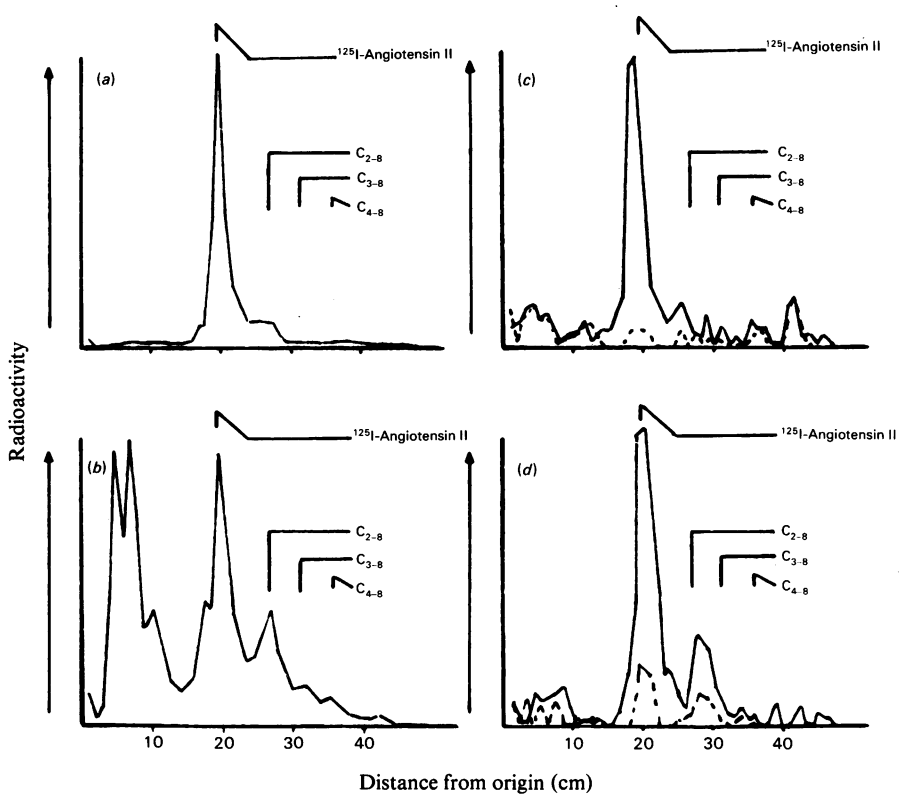


Fig. 2. *Chromatographic analysis of free and bound radioactivity*
Incubation was for 60min at 22°C. (*a*) Control incubation, no membrane material; (*b*) free radioactivity after incubation with 93 $\mu$g of membrane protein/200 $\mu$l; (*c*) total bound radioactivity after incubation with 0.2nM-$^{125}$I-angiotensin II; (*d*) total bound radioactivity after incubation with 20nM-$^{125}$I-angiotensin II. The broken lines in (*c*) and (*d*) denote non-specifically bound radioactivity determined in parallel incubations. The running positions of the *C*-terminal hepta- ($C_{2-8}$), hexa- ($C_{3-8}$) and penta- ($C_{4-8}$) peptides are indicated.

Table 1. *Effect of proteinase inhibitors on binding and degradation of $^{125}$I-angiotensin II*
Incubation was for 20min at 22°C, initial tracer concentration 0.3nM. Concentrations of inhibitors: EDTA, 5mM; EGTA, 5mM, dithiothreitol, 0.5mM; glucagon, 10 $\mu$M; Tos-Phe-$CH_2Cl$, 0.5mM; soya bean trypsin inhibitor (STI) 15 $\mu$M; Trasylol, 100k.-i.u./ml; phenylmethanesulphonyl fluoride (PMSF), 0.1mM; di-isopropyl phosphate, 20mM; $HgCl_2$, 1mM. Effects on binding were determined in triplicate (given as mean $\pm$ 1 S.D.); effects on tracer degradation were determined chromatographically by calculation of peak areas.

| Inhibitor | Binding (% of control) | Apparent rate constant for tracer decay (% of control) |
|---|---|---|
| EDTA | 57 $\pm$ 9 | 91 |
| EGTA | 81 $\pm$ 8 | 89 |
| Dithiothreitol | 67 $\pm$ 5 | 100 |
| PMSF/glucagon | 112 $\pm$ 10 | 75 |
| Tos-Phe-$CH_2Cl$ | 86 $\pm$ 4 | 75 |
| STI | 113 $\pm$ 5 | 95 |
| Trasylol | 105 $\pm$ 5 | 94 |
| Trasylol/Tos-Phe-$CH_2Cl$/STI/glucagon | 76 $\pm$ 4 | 71 |
| Trasylol/Tos-Phe-$CH_2Cl$/STI/glucagon/PMSF | 77 $\pm$ 5 | 54 |
| Di-isopropyl phosphite | 37 $\pm$ 11 | 11 |
| $HgCl_2$ | Not done | 0 |

Table 2. *Accuracy of the filtrate immunoassay*
$^{125}$I-angiotensin II was mixed with a completely metabolized tracer preparation (see the text) such that $^{125}$I-angiotensin II represented varying proportions of the total radioactivity. (*a*) Expected proportion; (*b*) proportion as determined by immunoassay (mean of six determinations ± 1 S.D.); (*c*) proportion as determined chromatographically by calculation of peak areas.

| $^{125}$I-angiotensin II (% of total radioactivity) | | |
|---|---|---|
| (*a*) Expected | (*b*) Immunoassay | (*c*) Chromatographic |
| 100 | 100 | 96 |
| 75 | 78 ± 10 | 75 |
| 50 | 47 ± 10 | 54 |
| 20 | 18 ± 8 | 26 |
| 0 | −8 ± 5 | < 10 |

without tracer angiotensin II prior to incubation with 0.3 nM-$^{125}$I-angiotensin II for 60 min at 22°C. Binding capacity decreased exponentially with an 8% loss after 60 min preincubation at 22°C and a 30% loss after 60 min at 37°C. No change in binding capacity was observed after 4 h preincubation on ice.

Specific binding of $^{125}$I-angiotensin II to the membrane fraction reached an apparent plateau within 50 min (Fig. 3*a*) of incubation, depending on tracer concentration, and started to decline after 70–90 min due to tracer degradation. In all such experiments tracer angiotensin II concentration decayed in a simple exponential manner. Dissociation profiles of specifically bound $^{125}$I-angiotensin II from the membrane fraction were identical in the presence of 0 and 5 μM-angiotensin II and deviated markedly from simple exponential decay, indicating heterogeneity of binding sites and the absence of site–site co-operative effects (Fig. 3*b*).

Binding was saturable but only at $^{125}$I-angiotensin II concentrations which were extremely high in relation to plasma angiotensin II levels in the rat (Fig. 4). Although not quantitatively applicable, Scatchard transformations of the binding data (Fig. 4, inset) showed marked curvature consistent with the presence of two classes of binding sites. Application of direct curve-fitting methods to these results allowed determination of forward rate constants and binding site concentrations for two classes of binding sites, but the reverse rate constants were poorly estimated. Computer simulations indicated that the shape of such curves is influenced to a relatively small extent by changes in dissociation rates. Analysis of saturation and dissociation curves together permitted determination of all binding constants (Table 3). To allow determination of all binding constants from a single experiment, combined saturation and dissociation experiments were performed (Fig. 5). In these later experiments (see the Discussion), the Scatchard transformations of the binding data were linear, indicating the presence of a single class of binding sites, although the dissociation profiles still deviated from simple exponential decay. Analysis showed no change in the affinities of the two

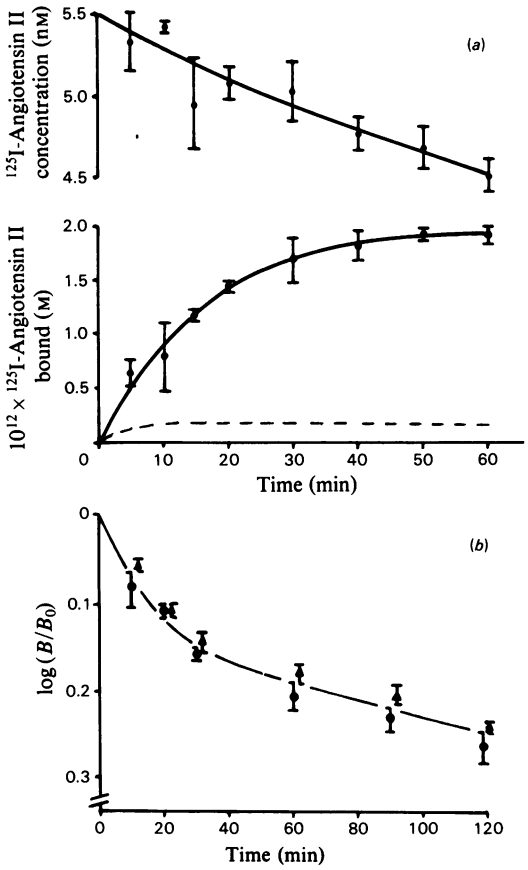


Fig. 3. *Binding and degradation of $^{125}$I-angiotensin II as a function of time at 22°C*
(*a*) Incubation comprised 27 μg of membrane protein/200 μl, initial tracer concentration 0.55 nM. Points indicate mean ± 1 S.D. for three or four determinations. The broken line denotes predicted low-affinity component of binding. (*b*) Dissociation of specifically bound radioactivity as a function of time. Membrane fraction material was incubated with 0.53 nM-$^{125}$I-angiotensin II for 60 min at 22°C, then the forward reaction was blocked by dilution (1:32) (▲) or addition of unlabelled angiotensin II (to 5 μM) (●) and the incubation was continued for the times indicated prior to determination of bound tracer. Points denote mean ± 1 S.D. for four to six determinations (log scale).

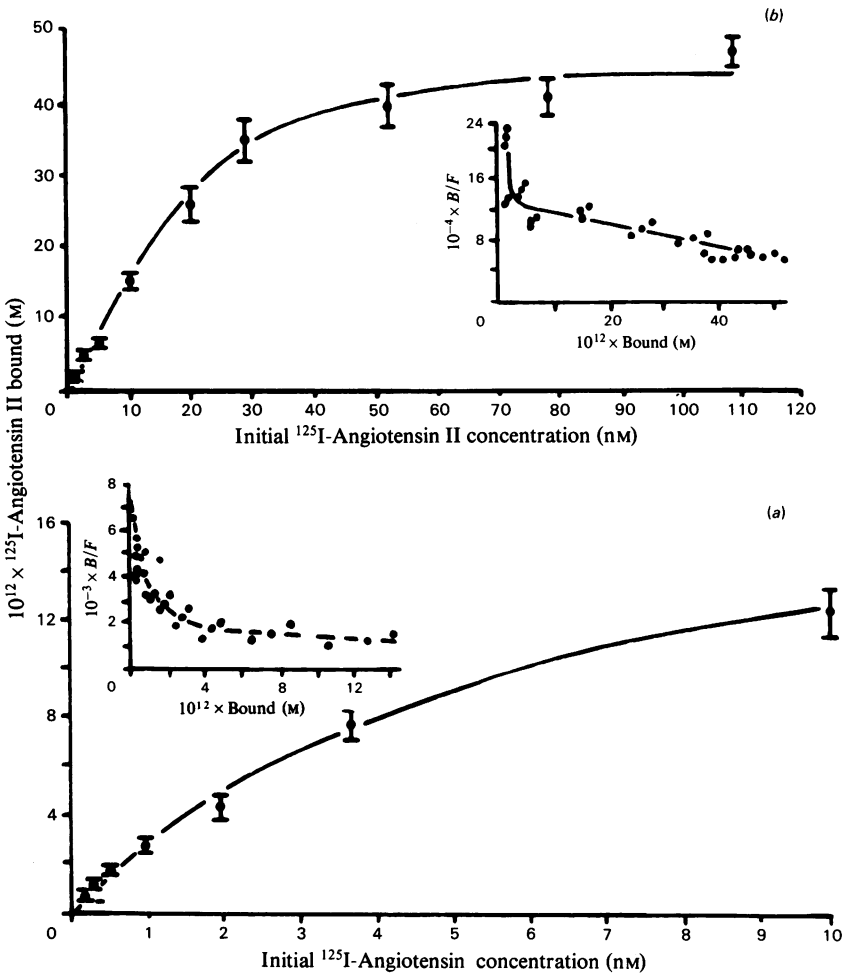


Fig. 4. *Binding of $^{125}$I-angiotensin II as a function of initial tracer concentration over two concentration ranges* (*a*) 0–10nM; (*b*) 0–110nM. Incubation was for 60min at 22°C, membrane protein concentration in (*a*) 27μg/200μl, in (*b*) 18μg/200μl. Points denote mean ± 1S.D. for three determinations. Insets show Scatchard transformations of the binding data, each point corresponding to an individual determination.

classes of binding sites, but a 2–3-fold increase in concentration of high-affinity sites (Table 3). This was due to a change in the method of preparing the arterial membrane fraction which prevented heating of the tissue during homogenization. Over the range of tracer concentrations employed for these experiments (0–10nM), binding to the low-affinity site is virtually linear with tracer concentration (Fig. 5, broken line), resulting in poor estimation of the binding constants for these sites. Modification of the homogenization procedure did not affect the kinetics of ligand degradation. For all experiments the rate constant for $^{125}$I-angiotensin II degradation was linearly related to membrane protein concentration, with a slope of $(7.5 \pm 0.7) \times 10^{-7} s^{-1} \cdot mg^{-1} \cdot l$ (correlation coefficient 0.94, $n = 18$).

Based on the kinetic constants determined from saturation binding curves, the low-affinity component of binding in the time course experiments may be reasonably approximated by a straight line parallel to the abscissa (Fig. 3*a*, broken line). This approximation was necessary in order to analyse the binding data from such experiments, using a four-parameter fitting procedure (three parameters relating to the high-affinity site and the ordinate intercept for the linear component of binding). This approach yielded kinetic constants for the high-affinity site agreeing well with those determined from saturation curves (Table 3).

Table 3. *Binding constants derived by nonlinear regression analysis of (a) separate saturation (n = 4) and dissociation (n = 4) experiments; (b) combined saturation/dissociation experiments (n = 4); and (c) association time course experiments (n = 3)*

In (*b*) the parameters relating to the low-affinity site were poorly estimated and confidence limits are not given. A parameter estimate differing significantly from that in column (*a*) is indicated (*). Binding constants for $^{125}$I-angiotensin II derived from analysis of competitive ligand binding curves are given in Table 4. The increase in the concentration of high-affinity sites (column *b*) is discussed in the text. Abbreviation used: 95% C.I., approx. 95% confidence limits.

| | | High-affinity site | | | | | |
|---|---|---|---|---|---|---|---|
| | | (*a*) | | (*b*) | | (*c*) | |
| Parameter | Unit | Estimate | 95% C.I. | Estimate | 95% C.I. | Estimate | 95% C.I. |
| Forward rate constant | $M^{-1} \cdot s^{-1}$ | $1.3 \times 10^6$ | $2.0 \times 10^6$<br>$8.6 \times 10^5$ | $8.6 \times 10^5$ | $1.1 \times 10^6$<br>$6.7 \times 10^5$ | $1.8 \times 10^6$ | $2.7 \times 10^6$<br>$1.2 \times 10^6$ |
| Reverse rate constant | $s^{-1}$ | $4.6 \times 10^{-5}$ | $7.6 \times 10^{-5}$<br>$2.8 \times 10^{-5}$ | $4.2 \times 10^{-5}$ | $6.5 \times 10^{-5}$<br>$2.7 \times 10^{-5}$ | $4.3 \times 10^{-5}$ | $8.3 \times 10^{-5}$<br>$2.2 \times 10^{-5}$ |
| Binding sites | fmol/mg of protein | 35 | 48<br>23 | *79 | 87<br>71 | 37 | 41<br>34 |
| $K_d$ | pM | 35 | 74<br>17 | 49 | 78<br>31 | 24 | 56<br>12 |

| | | Low-affinity site | | | | | |
|---|---|---|---|---|---|---|---|
| | | (*a*) | | (*b*) | | (*c*) | |
| Parameter | Unit | Estimate | 95% C.I. | Estimate | 95% C.I. | Estimate | 95% C.I. |
| Forward rate constant | $M^{-1} \cdot s^{-1}$ | $1.1 \times 10^5$ | $3.9 \times 10^5$<br>$3.0 \times 10^4$ | $2.6 \times 10^4$ | –<br>– | –<br>– | –<br>– |
| Reverse rate constant | $s^{-1}$ | $6.1 \times 10^{-3}$ | $1.8 \times 10^{-2}$<br>$2.1 \times 10^{-3}$ | $1.3 \times 10^{-3}$ | –<br>– | –<br>– | –<br>– |
| Binding sites | fmol/mg of protein | 680 | 1200<br>150 | 868 | –<br>– | –<br>– | –<br>– |
| $K_d$ | nM | 55 | 240<br>13 | 50 | –<br>– | –<br>– | –<br>– |

Specificity of the high-affinity site for angiotensin II was demonstrated by displacement of $^{125}$I-angiotensin II by angiotensin and unrelated peptides (Fig. 6). Under the conditions employed (30 min incubation, initial tracer concentration 0.3 nM) over 95% of the control bound tracer is associated with the high-affinity site. The affinity series after correction for tracer and competing ligand degradation was $^{125}$I-angiotensin II = angiotensin II > angiotensin III > [Sar$^1$,Ile$^8$]angiotensin II > [Sar$^1$,Gly$^8$]angiotensin II ≫ angiotensin I = bradykinin = arginine-vasopressin. The Sar$^1$ peptides were found to be resistant to degradation. In the case of angiotensin I, binding constants could not be calculated, as angiotensin II production during incubation accounted for almost all of the observed displacement. The computed affinities of the angiotensin peptides are given in Table 4. The kinetic constants for $^{125}$I-angiotensin II were in good agreement with those derived by the more direct methods described above.

$Na^+$ increased binding at 50 mM and 100 mM but had no effect at normal plasma concentrations. In the presence of $Na^+$, $K^+$ had a biphasic effect, increasing binding at 2 mM (110% of control) and decreasing binding at 5 mM (90% of control). Substitution of $Li^+$ for $Na^+$ resulted in a fall in binding to around 60% of control at all concentrations, but $K^+$ in the presence of $Li^+$ similarly produced a biphasic effect. A comparable, though less marked, pattern was shown by $NH_4^+$ in the presence of $Na^+$. In the absence of other metal ions, $K^+$ increased binding with a plateau at 10 mM. $Mg^{2+}$ and $Mn^{2+}$ were more effective, but $Ca^{2+}$ was most potent, increasing binding to 180% of control at 10 mM (Fig. 7). The chelating agents EDTA and EGTA were inhibitory, reducing binding to 57% and 81% of control respectively at 5 mM. Dithiothreitol was also inhibitory at 0.5 mM (67% of control) (Table 1). Cysteine at 10 mM produced a marked fall in binding capacity, reaching a plateau at 25% of control after 60 min preincubation, and

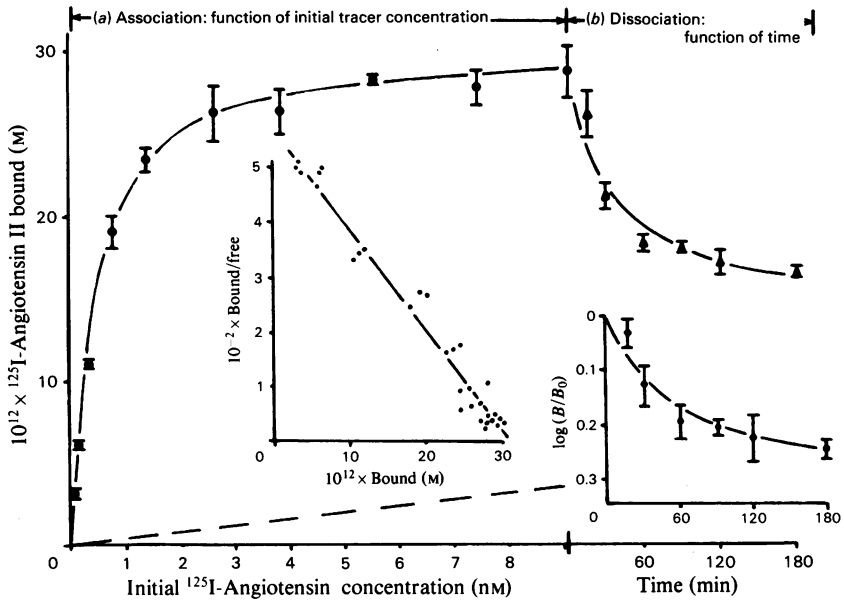


Fig. 5. *(a) Binding of $^{125}$I-angiotensin II as a function of initial tracer concentration and (b) dissociation of specifically bound tracer determined in one experiment*

Incubation was for 60min at 22°C, membrane protein concentration was 85μg/200μl. Points denote mean of three determinations ± 1S.D. (*a*) Inset shows Scatchard transformation of the binding data; the broken line denotes predicted low-affinity component of binding; (*b*) Inset shows log transformation of dissociation data. From the Scatchard plot the $K_d$ (calculated as $-1$/slope) is 0.54nM.

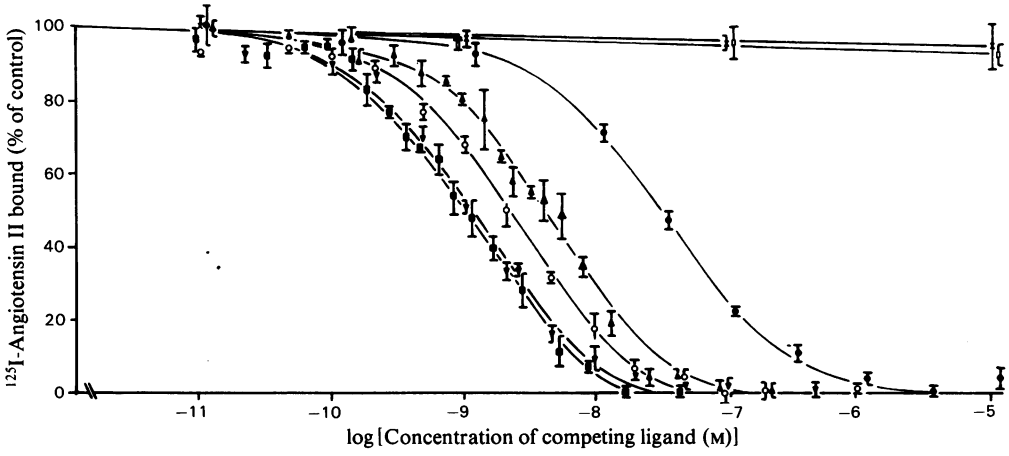


Fig. 6. *Displacement of $^{125}$I-angiotensin II by angiotensin and unrelated peptides*

Incubation was for 30min at 22°C, initial tracer concentration 0.3nM. Points denote mean of two to four determinations ± 1S.D. ■, Angiotensin II; ▲, des-Asp$^1$-angiotensin II; ●, angiotensin I; ▼, [Sar$^1$,Ile$^8$]angiotensin II; ○, [Sar$^1$,Gly$^8$]angiotensin II; ×, bradykinin; □, arginine-vasopressin.

the kinetics of $^{125}$I-angiotensin II binding in the presence of 10mM-cysteine suggested rapid destruction of binding sites.

## Discussion

The high-affinity binding sites identified in this study possess the properties expected of a physiological receptor for angiotensin. The kinetic constants should allow rapid interaction, and thus rapid response, with angiotensin II at normal plasma concentrations, and the observed concentration of binding sites (80fmol/mg of membrane protein) would correspond to a receptor density of the order of $10^4$ sites/cell (assuming $10^6$ cells/100mg wet wt. of tissue and 50% recovery in

Table 4. *Binding constants of angiotensin-related peptides determined by nonlinear regression analysis of the data depicted in Fig. 6*

In certain cases the reverse rate constants were poorly estimated and confidence limits are not given. A parameter estimate differing significantly from that for $^{125}$I-angiotensin II is indicated (*). Abbreviation used: 95% C.I., approx. 95% confidence limits.

| | Forward rate constant ($M^{-1} \cdot s^{-1}$) | | Reverse rate constant ($s^{-1}$) | | $K_d$ (pM) | |
|---|---|---|---|---|---|---|
| Peptide | Estimate | 95% C.I. | Estimate | 95% C.I. | Estimate | 95% C.I. |
| $^{125}$I-Angiotensin II | $1.2 \times 10^6$ | $1.7 \times 10^6$ | $4.9 \times 10^{-5}$ | $1.0 \times 10^{-4}$ | 41 | 66 |
| | | $8.9 \times 10^5$ | | $2.4 \times 10^{-5}$ | | 25 |
| Angiotensin II | $8.6 \times 10^5$ | $1.4 \times 10^6$ | $4.8 \times 10^{-5}$ | – | 56 | – |
| | | $5.4 \times 10^5$ | | – | | – |
| Angiotensin III | *$3.2 \times 10^5$ | $3.2 \times 10^5$ | $6.9 \times 10^{-5}$ | – | 219 | – |
| | | $3.2 \times 10^5$ | | – | | – |
| [Sar$^1$,Ile$^8$]Angiotensin II | *$4.2 \times 10^5$ | $7.3 \times 10^5$ | $2.7 \times 10^{-4}$ | – | 634 | – |
| | | $2.5 \times 10^5$ | | – | | – |
| [Sar$^1$,Gly$^8$]Angiotensin II | *$1.2 \times 10^5$ | $2.0 \times 10^5$ | *$9.6 \times 10^{-4}$ | $1.3 \times 10^{-3}$ | *8000 | 12000 |
| | | $7.0 \times 10^4$ | | $7.0 \times 10^{-4}$ | | 5300 |

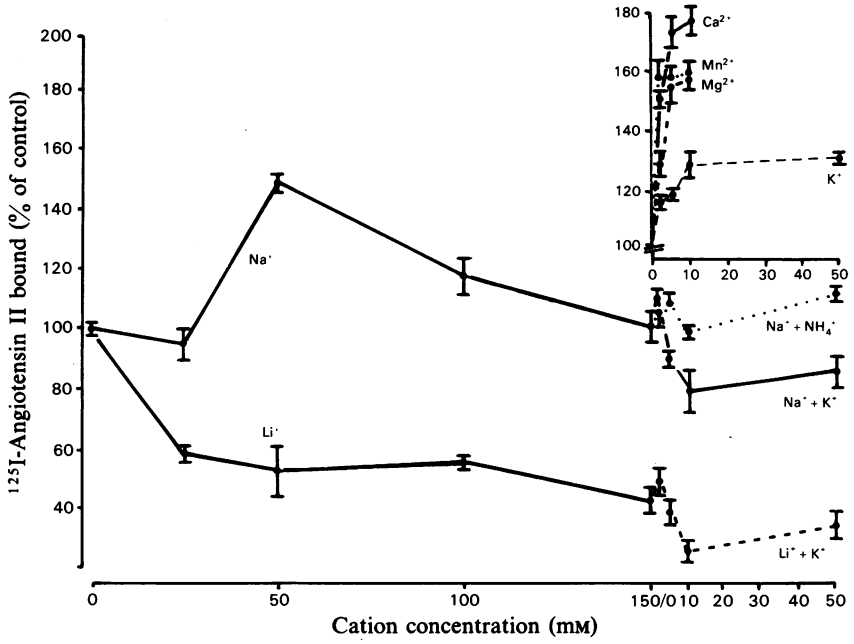


Fig. 7. *Effect of mono- and divalent cations (as chloride salts) on binding of $^{125}$I-angiotensin II*

Incubation was for 15 min at 22°C, initial tracer concentration was 0.3 nM. Main figure shows effect of $Na^+$ and $Li^+$, and effect of $K^+$ or $NH_4^+$ in the presence of $Na^+$ and effect of $K^+$ in the presence of $Li^+$. Inset shows effect of $K^+$, $Mg^{2+}$, $Mn^{2+}$ and $Ca^{2+}$ individually. Points denote mean of three to six determinations ± 1 S.D.

the preparation of the membrane fraction). Ketelslegers *et al.* (1975) have shown from computer modelling experiments that radioligand degradation in binding studies usually causes underestimation of affinity, while measurements of binding site concentration are relatively unaffected. From our experimental data and computer simulations (results not shown) we can confirm this finding. It is possible that the lower affinity ($K_d$ 1 nM) for the angiotensin receptor in this tissue reported by

Gunther *et al.* (1980) may have been due to unrecognized tracer angiotensin II degradation. The binding site concentration they quote (54 fmol/mg of protein) is similar to our value, and if our experimental results are analysed without compensation for tracer degradation (e.g. by Scatchard analysis, see Fig. 5 inset) the calculated $K_d$ is in the region of 1 nM.

The discrimination between angiotensin I, angiotensin II and des-Asp$^1$-angiotensin II (angiotensin III) is consistent with the pressor activities of these peptides in the rat, angiotensin I having little intrinsic activity (Bumpus *et al.*, 1961) and angiotensin III having approx. 30% of the activity of angiotensin II (Schwyzer, 1963; Carey *et al.*, 1978). [Sar$^1$,Ile$^8$]angiotensin II appeared equipotent with angiotensin II in displacing $^{125}$I-angiotensin II from the high-affinity site (Fig. 6). However, unlike the natural peptides, the Sar$^1$-substituted analogues were resistant to degradation. Consequently, the angiotensin II and angiotensin III displacement curves are shifted leftwards when correction for degradation of these peptides is made, whereas the angiotensin I displacement curve is shifted rightwards when angiotensin II production during incubation is taken into account. The affinity series therefore becomes $^{125}$I-angiotensin II = angiotensin II > angiotensin III > [Sar$^1$,Ile$^8$]angiotensin II > [Sar$^1$,Gly$^8$]angiotensin II $\gg$ angiotensin I = arginine-vasopressin = bradykinin (Table 4). The [Sar$^1$,Ile$^8$] peptide has both the highest affinity and the highest degree of agonist activity of all angiotensin II antagonists tested to date (Davis *et al.*, 1974). Since high plasma concentrations of antagonists (relative to plasma angiotensin II levels) are required to block pressor response to angiotensin II (Pettinger *et al.*, 1975), it would appear that the antagonists have an appreciably lower affinity than does angiotensin II. The observed affinity series is therefore consistent with results of pharmacological studies on the vascular angiotensin receptor. Although iodination of angiotensin II results in a partial loss of biological activity (Papadimitriou & Worcel, 1974) the iodinated and native peptides showed equal affinity for the receptor, as has been reported by Gunther *et al.* (1980) for this tissue and by Glossman *et al.* (1974) for the bovine adrenal cortex angiotensin receptor.

The effects of $Na^+$ and $K^+$ on tracer angiotensin II binding may relate to the action of angiotensin II in stimulating $Na^+$ and $K^+$ flux across the uterine plasma membrane (Hamon & Worcel, 1979). An effect of $Na^+$ concentration on angiotensin binding has also been reported in the zona glomerulosa (Douglas *et al.*, 1978), brain (Bennet & Snyder, 1980), renal brush border membranes (Brown & Douglas, 1982) and in isolated renal tubules where binding of angiotensin II was associated with changes in $Na^+$ fluxes (Freedlender & Goodfriend, 1977). Wright *et al.* (1982) have also reported $Na^+$ to stimulate $^{125}$I-angiotensin II binding in the rat mesenteric artery through an increase in receptor affinity.

The inhibitory action of the thiol-reducing agent dithiothreitol on binding, also noted by Gunther *et al.* (1980), may explain the specific inhibition of contractile response to angiotensin II in aorta by dithiothreitol and *N*-ethylmaleimide (Fleisch *et al.*, 1973). Cysteine caused a rapid fall in binding capacity, apparently through destruction of binding sites, or possibly by conversion of high-affinity sites to low-affinity sites. This is consistent with the presence of one or more essential disulphide bridges in the receptor molecule, and Capponi & Catt (1980) have reported the dog adrenal and uterine angiotensin receptors to be dimeric proteins with a subunit $M_r$ of 65000 in the adrenal and 68000 in the uterus.

It is unlikely that the second class of binding sites are involved in mediating response to angiotensin as their low affinity ($K_d$ 55 nM) would probably not permit interaction with angiotensin II at physiological concentrations. It is also unlikely that these sites represent binding of tracer to a peptidase enzyme, as tracer degradation was unaffected by the presence of 10 $\mu$M unlabelled angiotensin II which blocks high- and low-affinity binding. The low capacity (680 fmol/mg of membrane protein) and saturability is inconsistent with uptake of radioactivity into membrane vesicles. Furthermore, chromatographic analysis of bound radioactivity after incubation with 20 nM-$^{125}$I-angiotensin II, where a significant proportion (20%) of the bound radioactivity is associated with the low-affinity site, showed it to be composed entirely of $^{125}$I-angiotensin II and (to a lesser extent) $^{125}$I-angiotensin III (Fig. 2*d*). The low-affinity site therefore appears to discriminate between physiologically active angiotensin peptides and the inactive metabolites produced during incubation.

In the earlier saturation binding experiments (Fig. 4) the concentration of high-affinity sites was reduced, and the low-affinity component of binding more evident. Therefore, we have presented these results since they constitute important evidence for the presence of two classes of binding sites. In the later experiments (Fig. 5) the increased concentration of high-affinity sites masked the low-affinity component of binding in the saturation curves, but the dissociation curves still deviated from simple exponential decay. In all cases, dissociation profiles were identical in the presence of 0 and 5 $\mu$M unlabelled angiotensin II, demonstrating the absence of site–site co-operative effects.

The binding constants for the high-affinity site derived from three experimental approaches are in good agreement and should allow rapid interaction with angiotensin II at physiological concentrations. The parameters relating to the low-affinity site tended to be poorly estimated due to the linearity of binding with tracer concentration, even at relatively high $^{125}$I-angiotensin II concentrations, and masking by the high-affinity component of binding. Four-parameter fitting, assuming a linear low-affinity component of binding, applied to saturation binding curves such as that shown in Fig. 5 yielded values for the high-affinity-site binding constants agreeing well with those given in Table 3, simplifies both the experimental procedures and the analysis of binding data, and may provide the basis of an assay for the high-affinity receptors.

Direct curve-fitting techniques, although computationally demanding, represent the optimum method for analysis of complex ligand binding systems such as that described. Correction for ligand and/or receptor degradation is preferable to the use of proteinase inhibitors, which may have a significant adverse effect on the receptor(s), and the flexibility of nonlinear regression will allow quantitative description of other factors, such as heterogeneity of binding sites or co-operative effects which may invalidate conventional methods of analysis. Even where linearizing transformations are technically applicable, simple curve-fitting procedures may be more suitable, as transformation can distort the experimental data (Duggleby, 1980; Thakur *et al.*, 1980). An advantage of the method we have used is that kinetic constants may be derived from a single binding isotherm. This is an important feature in respect of hormone–receptor systems, where *in vivo* a dynamic rather than true chemical equilibrium will prevail, and equilibration constants may be inappropriate for a true description of the system. Curve-fitting methods have been previously applied for kinetic analyses (Ketelslegers *et al.*, 1975).

In conclusion, we have identified angiotensin II binding sites in resistance vessels which display the properties expected of a physiological receptor, and which are likely to be important mediators of pressor responsiveness to angiotensin. The methods we have employed for the analysis of radioligand binding data should be applicable to other complex systems where conventional methods are invalid.

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# APPENDIX

## Statistical analysis of radioligand binding data

The data were analysed with a standard regression computer package [BMDP program PAR (derivative-free non-linear regression)], (Dixon, 1981) in conjunction with a FORTRAN program which numerically integrated the differential equations describing the reaction under study. For two classes of binding sites, with terms for decay of tracer and one class of sites, these are:

$$-d[H]/dt = k_{f_1}[H]\cdot[R_1] - k_{r_1}[HR_1] + k_{f_2}[H]\cdot[R_2] - k_{r_2}[HR_2] + k_{dH}[H]$$

$$-d[R_1]/dt = k_{f_1}[H]\cdot[R_1] - k_{r_1}[HR_1] + k_{dR}[R_1]$$

$$-d[R_2]/dt = k_{f_2}[H]\cdot[R_2] - k_{r_2}[HR_2]$$

$$d[HR_1]/dt = k_{f_1}[H]\cdot[R_1] - k_{r_1}[HR_1]$$

$$d[HR_2]/dt = k_{f_2}[H]\cdot[R_2] - k_{r_2}[HR_2]$$

where $k_{f_n}$ = forward rate constant
$k_{r_n}$ = reverse rate constant
$[R_n]$ = receptor concentration
$[HR_n]$ = concentration of H–R complex (bound)
} $n = 1$, high affinity site; $n = 2$, low affinity site

$[H]$ = radioligand concentration
$k_{dH}$ = rate constant for tracer decay
$k_{dR}$ = rate constant for $[R_1]$ decay

The rate constants for decay of tracer and the high-affinity site were taken as known, leaving four rate constants and two receptor concentrations to be estimated. Where results were amalgamated from several experiments, it was assumed that the receptor concentrations were proportional to protein, and the constants of proportionality were estimated. Where a linear low-affinity component of binding was assumed, the terms relating to the low-affinity site were omitted and a separate linear component estimated for each experiment. For analysis of competitive binding curves, the low affinity component of binding was ignored and the forward and reverse rate constants for tracer and competing ligands and the concentration of high-affinity sites estimated.

The analysis was performed on the assumption that the variance of the observations was homogeneous on the logarithmic scale, which corresponds to the assumption that the observations have a constant percentage variability.

It was found that the analysis was more stable numerically when the regression program was estimating the logarithms of the rate constants. Consequently, the program provided estimates and approximate standard deviations for the logarithms of the rate constants, and the estimates were then transformed back to their original scales.

The approximate 95% confidence limits for each parameter were calculated on a log scale as point estimate $\pm$ two standard deviations of the estimate, and the endpoints of the interval were then transformed back to their original scales.

The standard deviation for $\log (k_r/k_f)$, i.e. $\log (K_d)$ was calculated as:

$$\sqrt{(s_r^2 + s_f^2 - 2p_{rf} \cdot s_r \cdot s_f)}$$

where $s_r$ = asymptotic standard deviation for $\log (k_r)$

$s_f$ = asymptotic standard deviation for $\log (k_f)$

$p_{rf}$ = asymptotic correlation of $\log (k_r)$ with $\log (k_f)$

For any parameter, the difference in parameter estimates from two sets of experimental data was taken as significant when the difference between point estimates exceeded two standard deviations of the difference, i.e. when

$$(E_A - E_B) > 2\sqrt{(s_A^2 + s_B^2)}$$

where $E_A$ and $E_B$ are the point estimates

$s_A$ and $s_B$ are the corresponding standard deviations