0026-895X/89/060809-09$02.00/0

MOLECULAR PHARMACOLOGY, 35:809-817

Divalent Cation-Induced Interconversion of Hepatic Angiotensin Receptor Subtypes

JAMES MCQUEEN and PETER F. SEMPLE

Medical Research Council Blood Pressure Unit, Western Infirmary, Glasgow G11 6NT, United Kingdom

Received September 6, 1988; Accepted March 20, 1989

SUMMARY

The effects of divalent cations on the hepatic angiotensin receptor have been investigated using radioligand binding methods. With a plasma membrane-enriched fraction from rat liver, a total binding capacity for angiotensin peptides of 0.5 pmol/mg of membrane protein was observed. In the absence of divalent cations, almost all of these sites showed low affinity (K_D, 11.25 nM) for angiotensin II. In the presence of Ca^{2+}, there was a concentration-dependent increase in the proportion of sites with high affinity (K_D, 0.94 nM) for angiotensin II. Mg^{2+}, Sr^{2+}, and Ba^{2+} were less effective in this respect, although Mg^{2+} also modified affinity of the high affinity subtype. Monovalent cations (Na^+, Cs^+, and K^+) had little effect on angiotensin binding. Both receptor subtypes showed high and approximately equal affinity for sarcosine^1-analogues of angiotensin II and 10-fold lower and equal affinity or Ile^7-angiotensin III. The low affinity subtype appeared to be more sensitive to N-terminal deletions in the peptide. Interconversion of receptor subtypes could be prevented by 5′-guanylylimidodiphosphate, La^{3+}, diltiazem, and verapamil. The results show that the hepatic angiotensin receptor can exist in high and low affinity states *in vitro* and that the proportion in each state can be modified by divalent cations, guanine nucleotides, and some Ca^{2+} antagonist drugs.

With membrane fractions from many tissues, the binding of angiotensin II is markedly altered by cations. In vascular (1), nonvascular (2), and cardiac (3, 4) smooth muscle, divalent cations (Ca^{2+} and Mg^{2+} at 0–10 mM) increase the number of binding sites detected by radioligand receptor assay, whereas sodium usually shows only a modest effect. In contrast, the affinity of the adrenocortical and brain angiotensin receptors is substantially increased by sodium (0–150 mM) whereas divalent cations have little effect in these tissues (2, 5, 6). In renal tubules, both sodium and divalent cations influence angiotensin binding, sodium increased receptor affinity and divalent cations increasing receptor density (2, 7). Thus, there appear to be two effects, which vary in magnitude between tissues, one involving sodium and the other involving calcium and/or magnesium.

In other hormone receptor systems, a differential influence of cations on agonist and antagonist binding has been observed (8–12), an effect thought to involve a GTP-binding receptor-effector coupling protein (13). It has been suggested that the cation effects on the adrenocortical and hepatic angiotensin receptors are associated with such a GTP-binding protein (14, 15). However, in some cases no such differential modulation of ligand binding is seen for angiotensin analogues (6). *In vitro* studies with smooth muscle have indicated that desensitization of the tissue to angiotensin II occurs through a mechanism involving Ca^{2+} (16). We have recently shown that the effect of Ca^{2+} on the rat mesenteric artery angiotensin receptor is modified by manipulation of the plasma angiotensin II concentration before removal of the tissue and we have proposed that there is a cation-dependent stage in vascular receptor regulation (1).

To extend our observations made in rat mesenteric artery, we have examined the effects of cations on the hepatic angiotensin receptor. Results from previous studies (17) suggest that these effects are similar in both tissues, and solubilized angiotensin receptors from liver and uterine muscle show similar physicochemical characteristics (18, 19). Current evidence indicates that the same second messenger/intracellular signaling mechanism is present in smooth muscle cells and hepatocytes (20, 21). Therefore, the liver appears to provide an adequate model for vascular muscle in regard to angiotensin receptor biochemistry and physiology.

Materials and Methods

Tyrosyl ^{125}I-[Ile^5]angiotensin II (^{125}I-angiotensin II; 1880 Ci/mmol) and 1-[*4,6 propyl*-^{3}H]dihydroalprenolol ([^{3}H]dihydroalprenolol; 110 Ci/mmol) were obtained from New England Nuclear (Boston, MA) and Amersham International, respectively. Unlabeled peptides were from Peninsula Laboratories (San Carlos, CA) and Cambridge Biochemicals. Trifluoperazine dihydrochloride was from Aldrich Chemicals (Milwaukee, WI) and nitrendipine from Bayer Pharmaceuticals. Diltiazem HCl,

ABBREVIATIONS: BSA, bovine serum albumin; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Gpp(NH)p, 5′-guanylylimidodiphosphate; Sar, sarcosine, *N*-methylglycine; GTP-γ-S, guanosine 5′-*O*-(3-thiotriphosphate).

verapamil HCl, and other drugs and biochemicals were from Sigma Chemical Co. (St. Louis, MO).

Preparation of the liver membrane fraction. Livers were obtained from male Sprague-Dawley rats (325–350 g) and a semipurified plasma membrane fraction was prepared as previously described (22). The membrane pellet was suspended in 50 mM Tris·HCl, pH 7.4, 120 mM NaCl (Tris/NaCl) and aliquots were frozen in methanol/dry ice and stored at −70°. Angiotensin binding was stable for 2–3 months under these conditions and did not differ between freshly prepared and frozen membrane preparations. The membrane material was thawed at 4° and aliquots were dispensed into polycarbonate tubes for incubation. All steps were performed at 0–4°. Membrane protein concentrations were determined by the Lowry method with bovine albumin as standard.

Marker enzyme assays. Sodium-potassium ATPase, glucose-6-phosphatase, and cytochrome *c* oxidase were assayed as previously described (23–25). Activities are expressed relative to a crude particulate fraction obtained by centrifuging a sample of the tissue homogenate at 120,000 × *g* for 60 min, after removal of unbroken cells and debris at 500 × *g* for 10 min. Enzyme assays were performed on fresh (unfrozen) preparations.

Receptor binding assay. For angiotensin binding, incubations were performed in a volume of 400 μl containing 50 mM Tris·HCl, pH 7.4, 120 mM NaCl, 0.5% (w/v) BSA, 350 units/ml bacitracin, 400 μg of membrane protein, 50–100 pM ^{125}I-angiotensin II and varying concentrations (0 or 50 pM to 200 nM) of unlabeled [Ile^8]angiotensin II, and cations, nucleotides, or drugs as appropriate. Nonspecific binding was defined in the presence of 10 μM unlabeled angiotensin II. Incubations were performed for 60 min at 22° unless specified otherwise. Bound and free radioactivity were separated by diluting the incubation mixture in 7 ml of cold filtration buffer (50 mM Tris·HCl, pH 7.4, 120 mM NaCl, 0.05%, w/v, BSA) and filtering through Whatman GF/F glass fiber filters that were prewetted with 150 mM NaCl, 1% (w/v) BSA, 1% (v/v) Tween 20. The filters were rinsed with another 7 ml of filtration buffer and dried, and filter-trapped radioactivity was determined in a Nuclear Enterprises 1612 γ counter (90% efficiency).

Dihydroalprenolol binding was determined under the same conditions but without bacitracin. Saturation binding curves were constructed using varying concentrations of [^{3}H]dihydroalprenolol (50 pM to 50 nM) and nonspecific binding was defined in the presence of 25 μM DL-propranolol. Filter-trapped radioactivity was determined using LKB Optiphase Safe scintillant and an LKB 1217 RackBeta counter (60% efficiency).

Recovery of the membrane material on the filters was determined after incubation in Tris/NaCl without BSA or bacitracin. NaCl (0.9%, w/v) was substituted for all other buffers. Protein was digested from the filters with 1 ml of 1 M NaOH, debris was removed by centrifugation, and the protein content was assayed by the Lowry method.

Stability of ^{125}I-angiotensin II. Stability of angiotensin II during incubations was assessed by immunoassay (26) and by chromatography on a 30-cm Waters I-60 high performance liquid chromatography column, using 0.3 M sodium phosphate, pH 7.4, 0.01% (v/v) Tween 20, at 2 ml/min. Radioactivity was determined in 0.4-ml fractions.

Analysis of binding data. Results are presented as specific ligand binding, calculated as total minus nonspecific binding. Data were analyzed using a weighted least-squares nonlinear regression method, essentially as described previously (26, 27). Dissociation equilibrium constants (K_D) were determined on a logarithmic scale for reasons of numerical stability and also because these parameters have a more normal distribution when expressed in this way. Comparison of fitting models was made using an appropriate *F* test procedure (27). Scatchard plots are shown for illustrative purposes only.

Results

Composition of the membrane material. Subcellular marker enzyme assays indicated that the material used in the binding studies was predominantly plasma membrane. Enrichment factors relative to a crude particulate fraction (120,000 × *g* pellet) were 5.5–7.2 for ouabain-sensitive Na^+/K^+ ATPase, 0.8–1.5 for glucose-6-phosphatase, and 0.05–0.2 for cytochrome *c* oxidase (ranges for four separate preparations). The density of high affinity angiotensin binding sites was 6.2–7.0-fold higher in the semipurified preparation.

Effect of cations on angiotensin binding. The effects of various cations on binding of ^{125}I-angiotensin II and [^{3}H]dihydroalprenolol were examined (Fig. 1). Divalent cations markedly increased angiotensin binding; Ca^{2+} produced a 200% increase in binding with a half-maximal effect at 0.5 mM. Sr^{2+} and Ba^{2+} were equipotent and maximally produced half the stimulation of binding seen with Ca^{2+}. Monovalent cations K^+ and (not shown) Na^+ and Cs^+ (0–150 mM) had a negligible effect, increasing binding by about 30%. Chelating agents EGTA and EDTA decreased binding by about 20 and 40%, respectively. In contrast, divalent cations had a negligible effect on binding of [^{3}H]dihydroalprenolol. The control value for these experiments was defined in a 50 mM Tris·HCl buffer. Ca^{2+} had an identical effect when the buffer also contained 120 mM NaCl, and all subsequent experiments were performed with 50 mM Tris·HCl; 120 mM NaCl, pH 7.4. Specific binding of angiotensin II was 10–30% of total radioactivity, depending on Ca^{2+} concentration; nonspecific binding was 0.3–0.6% of total.

Membrane protein recovery on the filters was unaffected by Ca^{2+} or Mg^{2+} concentration (0–100 mM). Recovery was consistently greater than 90% and averaged 94.7 ± 4.0%. Concentrations above 100 mM resulted in a decrease in membrane recovery (and ligand binding), indicating a disruptive effect of these ions on membrane structure. This effect may initially diminish receptor binding without affecting membrane recovery and may explain the parallel 50% fall in angiotensin and dihydroalprenolol binding seen with 100 mM Ca^{2+} and Mg^{2+}.

Degradation of the tracer angiotensin II during incubation was assessed by immunoassay and liquid chromatography and was consistently less than 10%, irrespective of divalent cation concentration. Omission of bacitracin from the assay buffer resulted in extensive degradation, typically 70–80% in 0.5 mM Ca^{2+}.

Receptor assays were performed in 0 mM (10 mM EDTA plus 10 mM EGTA), 0.5 mM, and 10 mM Ca^{2+}. Scatchard analysis indicated a predominant effect of Ca^{2+} on receptor number but some change in affinity was also involved (Fig. 2A). Some curvature was evident (less so at high [Ca^{2+}]), indicating two or more classes of binding sites. However, when the effect of Ca^{2+} was expressed in a plot of bound against log(free) ligand concentration (not shown), very little change in binding was seen at saturating concentrations of angiotensin II. This suggested a reciprocal change in the numbers of high and low affinity binding sites, promoted by Ca^{2+}, which would be consistent with the changes in curvature in the Scatchard plots.

To test this possibility, nonlinear regression analysis was performed on the data using several models, and the goodness of fit values were compared (27). A two-site model (in which receptor concentrations but not affinities were allowed to vary) permitted a good fit to the data that was significantly better than that obtained with a one-site variable K_D model ($p < 0.001$), a one-site variable B_{max} model ($p < 0.001$), and a two-site model assuming no change in the low affinity site ($p = 0.007$). The residual sum of squares was not significantly im-

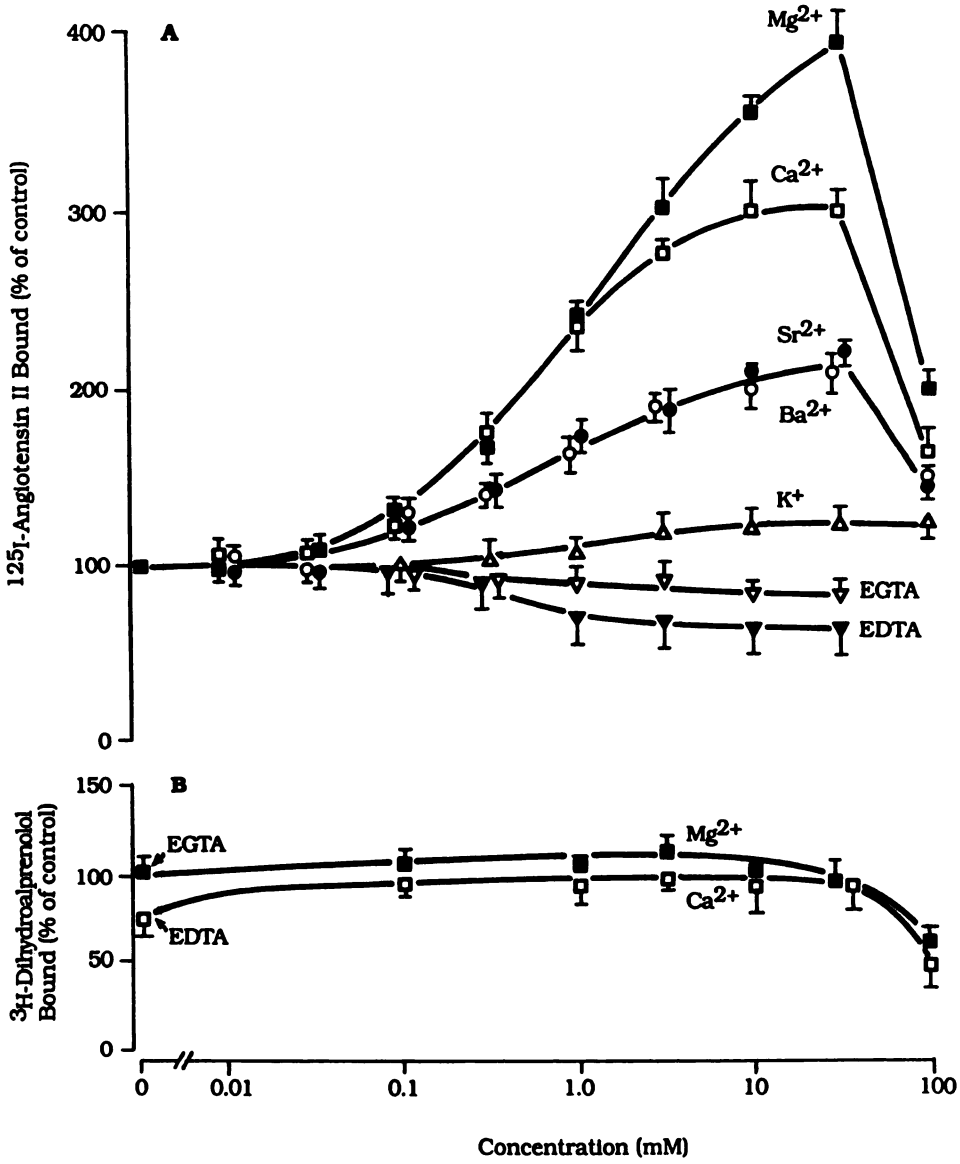

Fig. 1. Effect of monovalent and divalent cations and chelating agents on ^{125}I-angiotensin II binding (A) and [^{3}H]dihydroalprenolol binding (B). *Points* denote mean ± standard error for three experiments in triplicate. Control (100%) was defined in 50 mM Tris·HCl, pH 7.4. Cations were added as chloride salts, chelating agents as Na^+ salts. Tracer concentrations, 50 pM (A) and 100 pM (B).

proved (relative to the two-site model) by application of a three-site model ($p = 0.085$) or a two-site model allowing variation in both K_D and B_{max} values ($p = 0.140$). These tests were also applied to data obtained in the presence of 50 μM Gpp(NH)p and 400 μM diltiazem (see below) and the same conclusion was reached. Calcium, therefore, had a switching effect, converting low affinity sites (K_D, 11.25 nM) into a high affinity form (K_D, 0.94 nM) (Table 1).

There was no indication of a graduated change in receptor affinity with Ca^{2+}. However, to obtain a good fit to the data obtained in the presence of 0.5, 10, and 30 mM Mg^{2+} (Fig. 2B), it was necessary to allow for changes in receptor number and affinity. Magnesium appeared to promote conversion of low affinity sites to a high affinity form, but to a lesser extent than seen with Ca^{2+}. The K_D of the high affinity site in 0.5 mM Mg^{2+} was not different from that in any [Ca^{2+}] but was significantly less in 10 and 30 mM Mg^{2+}. The K_D for the low affinity site did not vary with Mg^{2+} concentration (Table 1).

Two classes of binding sites were observed for [^{3}H]dihydroalprenolol (K_D values of 1.80 and 330 nM). Neither was significantly affected by Ca^{2+} or Mg^{2+} (Table 1).

Specificity of receptor subtypes. To investigate the specificity of the high and low affinity receptor subtypes, displacement experiments were performed with various angiotensin analogues in 0 and 10 mM Ca^{2+}. Log-probit transformations of the results for the agonist peptides are shown in Fig. 3. The binding inhibition potencies were as expected, with Sar^1-angiotensin II > angiotensin II > angiotensin III > C-terminal (C_{3-8}) hexapeptide; rightward and leftward shifts were seen, indicating some difference in specificity of the two subtypes. Sar^1-angiotensin II and the antagonists Sar^1, Ile^8-angiotensin II, Sar^1, Thr^8-angiotensin II, and Ile^7-angiotensin III all showed a small but consistent rightward shift in the presence of Ca^{2+}, whereas a larger leftward shift was seen for angiotensin III and the C_{3-8} hexapeptide. Angiotensin I, Arg^8-vasopressin, and bradykinin (0.01–10 μM) produced less than 10% displacement of bound ^{125}I-angiotensin II (not shown). Because Ca^{2+} did not affect the affinity of either class of binding sites for angiotensin II, the data were analyzed assuming no change in affinity for the ligands under study. Simultaneous curve fitting was performed on all the data, a process involving estimating the K_D of each class of sites for the tracer and each competing ligand

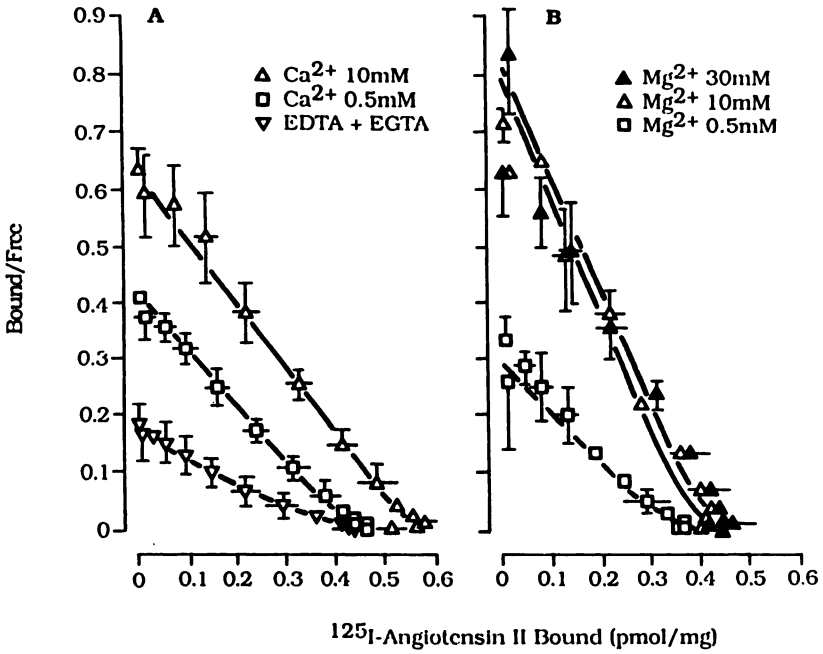

Fig. 2. Scatchard transformations of ^{125}I-angiotensin II binding data. A, Data were obtained in 0 mM Ca^{2+} (10 mM EDTA plus 10 mM EGTA), 0.5 mM Ca^{2+}, and 10 mM Ca^{2+}; *points* denote mean ± plus standard error for 10 experiments in duplicate. B, Data were obtained in 0.5, 10, and 30 mM Mg^{2+}; *points* are mean ± standard error for six experiments in duplicate. Computer-fitted lines based on the values in Table 1 are shown.

TABLE 1

Effect of Ca^{2+} and Mg^{2+} on binding of ^{125}I-angiotensin II and [^{3}H]dihydroalprenolol

Equilibrium binding constants (point estimate ± standard error) were obtained by nonlinear regression analysis of data obtained in Tris/NaCl buffer that contained 10 mM EDTA plus 10 mM EGTA or cations as indicated. K_D values are given in units of negative log molar, B_{max} values as pmol/mg of membrane protein. The number of replicate experiments is shown. Affinity constants for angiotensin II did not vary with the assay [Ca^{2+}] (see text).

Cation	Parameter	^{125}I-Angiotensin II Binding		No. of Experiments
		Receptor subtype A	Receptor subtype B	
All [Ca^{2+}]	K_D (−log M)	9.066 ± 0.064	7.873 ± 0.118	
EDTA + EGTA	B_{max} (pmol/mg)	0.062 ± 0.032	0.390 ± 0.030	10
Ca^{2+}, 0.5 mM	B_{max} (pmol/mg)	0.336 ± 0.024[a]	0.125 ± 0.025[a]	10
Ca^{2+}, 10 mM	B_{max} (pmol/mg)	0.539 ± 0.030[a]	0.021 ± 0.032[a]	10
Mg^{2+}, 0.5 mM	K_D (−log M)	8.988 ± 0.072	7.975 ± 0.390	6
	B_{max} (pmol/mg)	0.270 ± 0.041[a]	0.228 ± 0.078[a]	
Mg^{2+}, 10 mM	K_D (−log M)	9.318 ± 0.060[a]	8.072 ± 0.107	6
	B_{max} (pmol/mg)	0.366 ± 0.029[a,b]	0.156 ± 0.029[a,b]	
Mg^{2+}, 30 mM	K_D (−log M)	9.364 ± 0.079[a]	7.701 ± 0.164	6
	B_{max} (pmol/mg)	0.394 ± 0.028[a,c]	0.115 ± 0.029[a,c]	
Cation	**Parameter**	**[^{3}H]Dihydroalprenolol Binding**		**No. of Experiments**
		High affinity site	Low affinity site	
EDTA + EGTA	K_D (−log, M)	8.661 ± 0.079	6.334 ± 0.277	4
	B_{max} (pmol/mg)	58.9 ± 2.8	1960 ± 1160	
Ca^{2+}, 10 mM	K_D (−log M)	8.658 ± 0.190	6.456 ± 0.153	4
	B_{max} (pmol/mg)	57.6 ± 6.5	1670 ± 530	
Mg^{2+}, 10 mM	K_D (−log M)	8.912 ± 0.060	6.657 ± 0.072	4
	B_{max} (pmol/mg)	51.3 ± 1.3	1130 ± 130	

[a] Value differs significantly ($p < 0.05$) from that in EDTA plus EGTA.
[b] B_{max} differs significantly ($p < 0.05$) from that in the same [Ca^{2+}].
[c] B_{max} differs significantly ($p < 0.05$) from that in 10 mM Ca^{2+}.

and the binding site concentrations (Table 2). Neither class of binding sites discriminated between labeled and unlabeled angiotensin II. Lower affinities were noted for angiotensin III and the C_{3-8} hexapeptide, and the low affinity site appeared to be more sensitive to N-terminal deletions in the peptide; the K_D values differed 10-fold for angiotensin II, 20-fold for angiotensin III, and 50-fold for the C_{3-8} hexapeptide. This would account for the leftward shifts of the displacement curves for these ligands in the presence of Ca^{2+}. Those binding sites showing low affinity for angiotensin II displayed an equal or higher affinity for Sar^1-ligands (agonist and antagonists; K_D values only significantly different for Sar^1, Thr^8-angiotensin II), further suggesting that the N-terminus of the peptide is particularly important in determining binding to these sites. However, both classes of binding sites showed equal (but 10-fold lower) affinity for the heptapeptide antagonist Ile^7-angiotensin III. The receptor concentrations determined by this approach did not differ significantly from those in Table 1.

Effect of Guanine Nucleotides. Experiments were performed to determine whether the effect of Ca^{2+} on angiotensin binding involved guanine nucleotides. In the presence of 1.0 mM Ca^{2+}, GTP and nonhydrolyzable analogue Gpp(NH)p reduced angiotensin binding to the 0 mM Ca^{2+} (control) level, with ED_{50} values of 160 and 25 μM, respectively. ATP, cAMP,

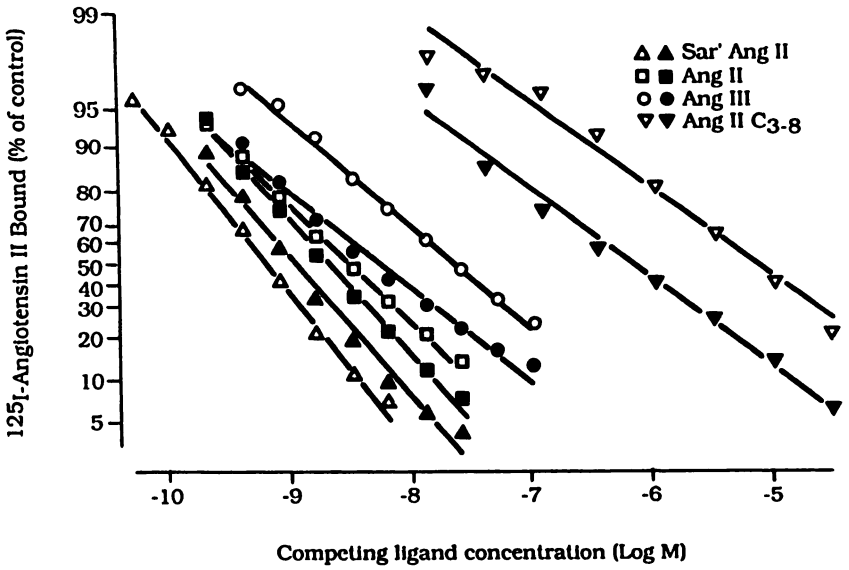

Fig. 3. Displacement of ^{125}I-angiotensin II by agonist fragments and analogues. Low-probit transformations are shown; points are mean of four experiments in triplicate. *Open symbols*, data obtained in 0 mM Ca^{2+} (10 mM EDTA plus 10 mM EGTA); *solid symbols*, data obtained in 10 mM Ca^{2+}. For clarity, error bars have been omitted and linear regression *lines* are shown. Tracer concentration, 100 pM. *Ang*, angiotensin.

TABLE 2

Specificity of the A and B angiotensin receptor subtypes

K_D values (units of negative log molar; point estimate ± standard error) for angiotensin-related peptides were obtained by nonlinear regression analysis of competition binding data (four replicate experiments with and without Ca^{2+} for each unlabeled ligand, nine points in triplicate in each).

Ligand	K_D	
	Receptor subtype A	Receptor subtype B
	−[log (M)]	
^{125}I-Angiotensin II	9.032 ± 0.163	8.023 ± 0.297[a]
Angiotensin II	9.033 ± 0.036	8.090 ± 0.128[a]
Angiotensin III	8.586 ± 0.046[b]	7.251 ± 0.205[a,b]
Angtiotensin II C_{3-8}	6.755 ± 0.327[b]	5.062 ± 0.024[a,b]
Sar^1-Angiotensin II	9.324 ± 0.045[b]	9.569 ± 0.266[b]
Sar^1, Ile^8-Angiotensin II	8.935 ± 0.029	9.637 ± 0.317[b]
Sar^1, Thr^8-Angiotensin II	8.711 ± 0.028[b]	9.287 ± 0.211[a,b]
Ile^7-Angiotensin III	8.315 ± 0.029[b]	8.159 ± 0.123[b]

[a] The K_D values for the high and low affinity sites are significantly different ($p < 0.05$).

[b] The K_D value is significantly different ($p < 0.05$) from that for angiotensin II.

and cGMP had little or no effect on angiotensin binding (results not shown). Identical effects have been described for the vascular angiotensin receptor (1). In the absence of divalent cations, no effect on binding was observed with any of these nucleotides.

Receptor assays were performed in 0, 0.5, and 10 mM Ca^{2+} and in the presence of 25, 50, and 100 μM Gpp(NH)p. A change in receptor affinity was expected, but the binding data obtained under these conditions were adequately described by the two-site model described above. The K_D values for the high and low affinity sites did not differ from those obtained in the absence of Gpp(NH)p ($p > 0.10$ in all cases). Ca^{2+} had a biphasic effect in the presence of 25 and 50 μM Gpp (NH)p, the number of high affinity sites being maximal in 0.5 mM Ca^{2+}. This biphasic effect was mirrored in the change in the number of low affinity sites (Fig. 4). In 100 μM Gpp(NH)p, the effect of Ca^{2+} on angiotensin binding was almost completely abolished. Therefore, there appeared to be two effects, a stimulatory effect of Ca^{2+} on binding that was itself inhibited by a process involving GTP. Inasmuch as no such biphasic pattern was seen in the absence of Gpp(NH)p, it appeared unlikely that the stimulatory effect of Ca^{2+} was due to cation-induced displacement and/or hydrolysis of endogenous GTP contamination of the membrane material. This view was supported by experiments in which the membrane material was preincubated at 22° or 37° in 0 and 10 mM Ca^{2+} and the effect of Ca^{2+} on angiotensin binding then determined (Fig. 5). At 22°, almost the same stimulation of binding was seen after prolonged (90 min) preincubation both in 0 and 10 mM Ca^{2+}. At 37°, binding declined by about 40% over 90 min of preincubation in 0 mM Ca^{2+} and by about 60% after 90 min in 10 mM Ca^{2+}. There was no selective effect of preincubation on angiotensin binding in 0 mM Ca^{2+} or in 10 mM Ca^{2+}, and the observed changes are more consistent with instability of the receptor.

Effect of Ca^{2+} antagonist drugs. Several Ca^{2+} antagonist drugs were examined for an effect on angiotensin binding (Fig. 6). $LaCl_3$ was most potent, blocking the stimulation of angiotensin binding by 1.0 mM Ca^{2+} with an ED_{50} of 70 μM. Diltiazem and verapamil at high concentrations had a similar effect (ED_{50} values of 450–600 μM). No effect was seen with nitrendipine and trifluoperazine. In 0 mM Ca^{2+}, no effect on angiotensin binding was observed with any of these compounds. Receptor assays were performed in 0, 0.5, and 10 mM Ca^{2+} and in the presence of 200, 400, and 800 μM diltiazem. The two-site model was found to provide a good fit to the data, and the K_D values for the high and low affinity subtypes did not differ from those determined in the absence of diltiazem ($p > 0.10$ in all cases). Diltiazem attenuated the Ca^{2+}-induced conversion of low affinity sites to a high affinity form. Verapamil and $LaCl_3$ were tested at concentrations of 800 and 200 μM, respectively, and found to have qualitatively the same effect (Fig. 7).

The inhibitory effect of diltiazem appeared to be noncompetitive with Ca^{2+}, because it could not be overcome by increasing the Ca^{2+} concentration (up to 100 mM). The ED_{50} for Ca^{2+} stimulation of angiotensin binding varied only slightly (0.5–0.8 mM) with increasing concentrations (0–800 μM) of diltiazem. The inhibitory effect of La^{3+} also appeared to be non-competitive with Ca^{2+} (not shown).

Discussion

In summary, a total binding capacity for angiotensin peptides of about 0.5 pmol/mg of membrane protein was observed in a

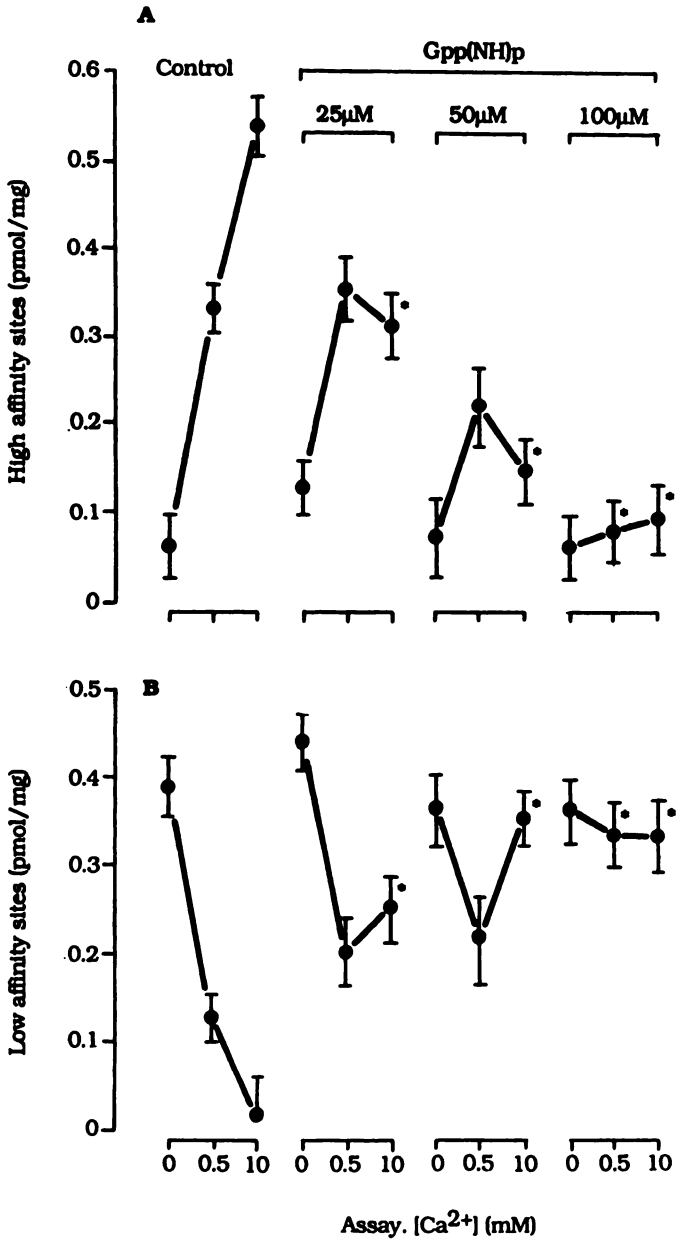

Fig. 4. Effect of Gpp(NH)p on ^{125}I-angiotensin II binding. Effect of Ca^{2+} (0, 0.5, and 10 mM) on numbers of high affinity (subtype A) (A) and low affinity (subtype B) (B) binding sites, in control experiments (10 experiments at each [Ca^{2+}]) and in the presence of 25, 50, and 100 μM Gpp(NH)p (four experiments at each [Ca^{2+}]) is shown. Receptor numbers are shown as point estimate ± standard error. *Parameter estimates significantly different from control ($p < 0.05$) in the same [Ca^{2+}].

plasma membrane fraction from rat liver. In the absence of divalent cations, almost all of these sites showed low affinity for angiotensin II (K_D, 11.25 nM; designated the B subtype). Ca^{2+} caused a concentration-dependent increase in the proportion of sites with high affinity (K_D, 0.94 nM; A subtype) for this peptide. The total binding site concentration varied only slightly (0.45–0.55 pmol/mg) under all conditions tested. Binding of the β-adrenergic antagonist [^{3}H]dihydroalprenolol was unaffected by divalent cations, demonstrating that this effect was not due to a nonspecific effect of Ca^{2+} on membrane structure. Both subtypes showed high and approximately equal affinity for Sar^1-angiotensin peptides and the B subtype was particularly sensitive to N-terminal deletions in the peptide, suggesting that the N-terminal structure of the peptide was of special importance in determining binding to the B subtype.

The cation and nucleotide effects reported here closely resemble those previously described for us for the vascular (rat mesenteric artery) angiotensin receptor (1, 26). Two classes of binding sites are present in this tissue but, due to technical difficulties, it was not possible to accurately quantify binding to the low affinity site. These similarities suggest that cation-promoted interconversion of receptor subtypes also occurs in vascular and perhaps other tissues, because low affinity angiotensin binding sites have been identified in various tissues (3, 6, 28, 29). Pharmacological data suggest that such interconversion occurs during smooth muscle response to angiotensin II (30).

Cations and guanine nucleotides modulate ligand binding in other receptor systems (8–12). In all cases, a differential effect on agonist and antagonist binding is seen, cations and/or nucleotides predominantly modifying agonist binding. These effects are thought to involve a GTP-binding protein that couples the receptor to its effector(s) (13). An apparently similar effect has previously been reported for the hepatic angiotensin receptor. Crane *et al.* (14) observed two classes of binding sites for angiotensin II (K_D values of 0.46 and 4.1 nM). In the presence of Mg^{2+}, 100 μM GTP-γ-S reduced the number of high affinity sites from 0.65 to 0.05 pmol/mg, without altering receptor affinity. Binding of the antagonist Sar^1, Ala^8-angiotensin II was unaffected by GTP-γ-S.

In this study, Gpp(NH)p similarly reduced the number of high affinity sites by preventing conversion of the B subtype to the A form. Because both subtypes showed approximately equal affinity for Sar^1-ligands and the total density of binding sites did not change, Ca^{2+} and Gpp(NH)p would have little effect on binding of these ligands. However, this effect was seen for Sar^1-angiotensin II [a potent agonist (31)], as well as the Sar^1-antagonists. Instead of a discriminatory effect on agonist and antagonist binding, the effects of Ca^{2+} and Gpp(NH)p appeared to depend on the N-terminal structure of the peptide. A similar observation has been made regarding the effect of Na^+ on angiotensin binding to brain and adrenocortical membranes (6).

An inhibitory effect of Gpp(NH)p on angiotensin binding has been reported for most tissues studied to date and may be associated with a GTP-binding coupling protein (13, 32). The effects of Gpp(NH)p on the adrenocortical receptor are agonist selective and characteristic of receptor/GTP-binding protein interactions (33). Divalent cations have little effect on angiotensin binding in this tissue (2, 5, 6). It is possible that in some tissues there may be two modulating systems acting on the receptor, one involving GTP that requires a cation as a cofactor and one involving (at least *in vitro*) only cations.

The biphasic effect of Ca^{2+} in the presence of Gpp(NH)p described here would be consistent with two processes acting on the receptor. The inhibitory effect of Ca^{2+} at concentrations above 0.5 mM may be due to receptor/GTP-binding protein interaction promoted by Gpp(NH)p. Thus, the potential for agonist-selective receptor interactions could exist (explaining the equal affinity of the A and B subtypes for the heptapeptide antagonist Ile^7-angiotensin III) but may be obscured by the higher affinity N-terminal-selective interaction seen with Sar^1-ligands. The increase in affinity of the A subtype with Mg^{2+}

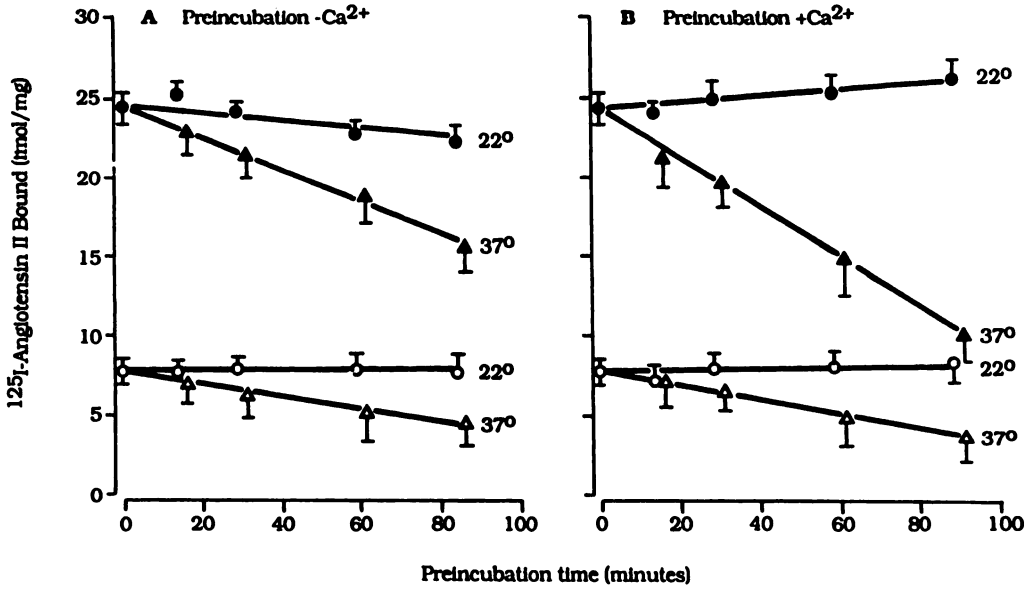

Fig. 5. Reversibility of the effect of Ca^{2+} on ^{125}I-angiotensin II binding. Samples of the membrane fraction (1 mg/ml) were preincubated in 0 mM Ca^{2+} (A) or 10 mM Ca^{2+} (B) at 22° or 37°. The membrane fraction was washed in Tris/NaCl that contained 25 mM EDTA and recovered by centrifugation and angiotensin binding was determined in 0 mM Ca^{2+} (*open symbols*) and 10 mM Ca^{2+} (*solid symbols*), using 30-min incubations at 22° and 50 pM tracer. *Points* denote mean ± standard error for three experiments in triplicate.

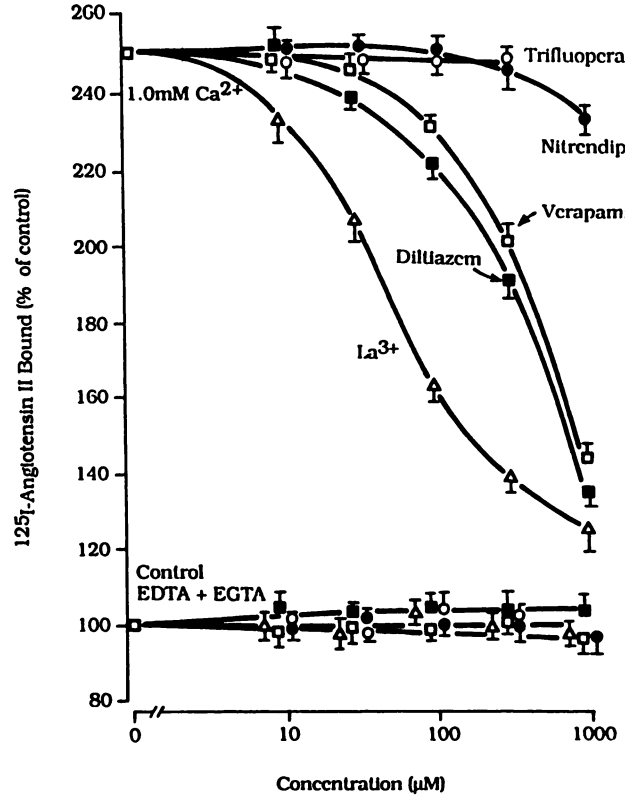

Fig. 6. Effect of Ca^{2+} antagonist drugs on ^{125}I-angiotensin binding in the absence and presence (1 mM) of Ca^{2+}. Nitrendipine was added as a 50 mM solution in ethanol, other drugs as a 50 mM solution in water. *Points* denote mean ± standard error for three experiments in triplicate. Tracer concentration, 50 pM.

may also be associated with a GTP-binding protein effect, because Mg^{2+} appears to be important for receptor/GTP-binding protein interactions (13). Because no biphasic effect of Ca^{2+} was seen in the absence of Gpp(NH)p and the effect of Ca^{2+} was reversible, it is unlikely that Ca^{2+} stimulation of angiotensin II binding was due to cation-induced displacement and/or hydrolysis of endogenous GTP contaminating the membrane material. We have previously found that Ca^{2+} and Gpp(NH)p modulate angiotensin binding in arterial membranes through apparently independent mechanisms (1).

As noted above, the A and B subtypes differ largely in their binding of N-terminal-modified angiotensin analogues. In smooth muscle tissues, desensitization to angiotensin II is accelerated by Sar^1-substitution and reduced by N-terminal deletions in the peptide (31, 34). Desensitization is also accelerated in low [Ca^{2+}] media (16), and tissue responsiveness to N-terminal-modified angiotensin analogues is differentially affected in low [Ca^{2+}] media and also by Ca^{2+} channel blockade (35). A Ca^{2+}-linked receptor-masking/unmasking effect (probably interconversion of high and low affinity receptor forms) occurs in vascular muscle in response to alterations in plasma angiotensin II concentration (1). Thus, there is evidence linking the Ca^{2+} effect described here with a mechanism determining tissue sensitivity to angiotensin II.

This report and the pharmacological studies cited above imply that desensitization is associated with ligand interaction with the B subtype and is promoted mainly by the N-terminal structure of the peptide. The latter point would suggest that Sar^1-antagonists can induce desensitization; these agents do show some noncompetitive inhibition (36). The two subtypes might arise through covalent or conformational changes in the receptor itself or interaction of the receptor with another factor. A tissue-specific regulatory factor would be consistent with the differing cation effects and regulatory changes (1, 2, 5, 37) occuring in (for example) vascular and adrenal tissue, while the receptors in these tissues (and liver) show similar physicochemical properties (18, 19). The Ca^{2+} concentrations used here much higher than those inside the cell, but observations made with a membrane fraction may not fully reflect the processes occuring in the intact cell. Evidence indicates that intracellular [Ca^{2+}] can alter angiotensin receptor status (35) and that interconversion of high and low affinity receptor subtypes occurs during tissue response to the peptide (30). Intracellular [Ca^{2+}] may serve as an integrator of hormonal stimuli in determining responsiveness to angiotensin.

The inhibitory effects of La^{3+}, diltiazem, and verapamil were clearly selective, because no effect of these compounds was seen in the absence of divalent cations. La^{3+} probably acts through

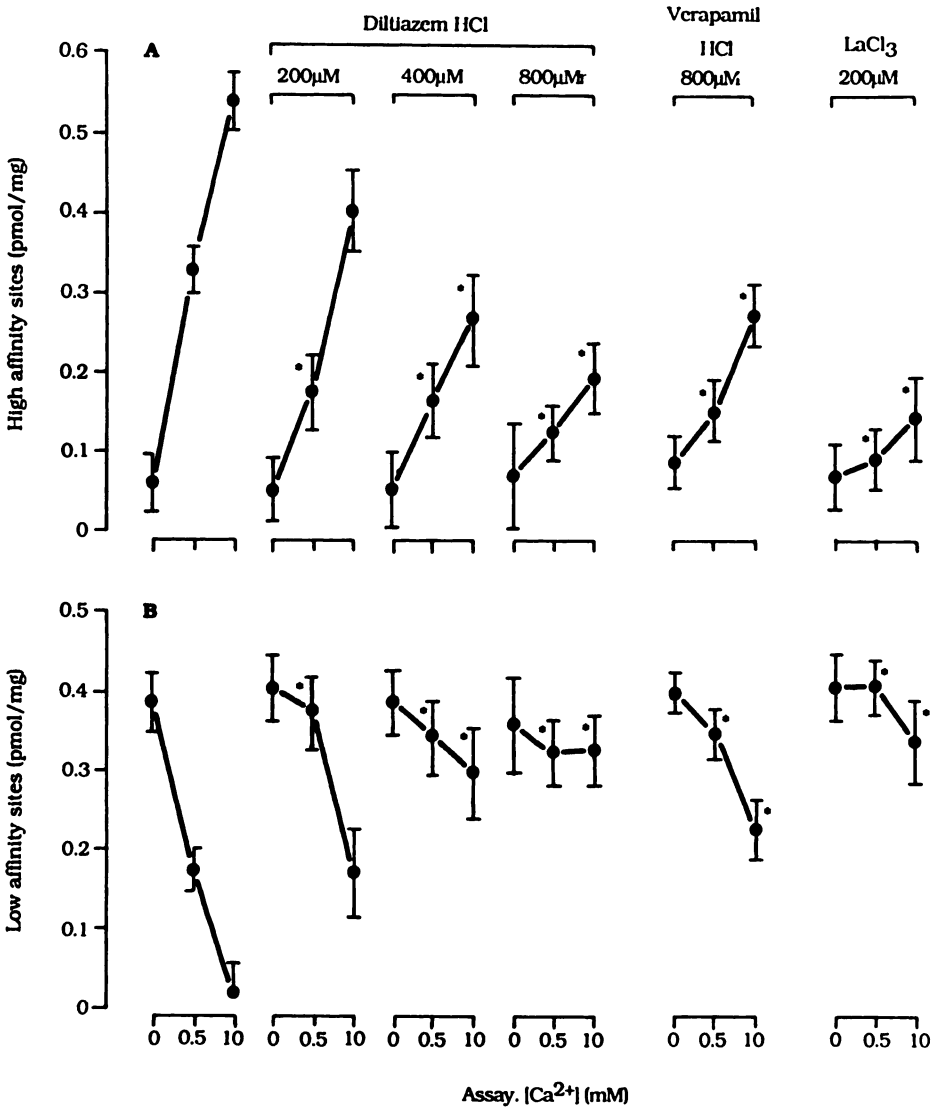

Fig. 7. Effect of Ca^{2+} antagonist drugs on ^{125}I-angiotensin II binding. Effect of Ca^{2+} (0, 0.5, and 10 mM) on numbers of high affinity (subtype A) (A) and low affinity (subtype B) (B) binding sites, in control experiments (10 experiments at each [Ca^{2+}]) and in the presence of diltiazem, verapamil, and $LaCl_3$ (four experiments at each [Ca^{2+}]) is shown. Receptor numbers are shown as point estimate ± standard error. *Parameter estimates significantly different from control ($p < 0.05$) in the same [Ca^{2+}].

direct blockade of Ca^{2+} binding sites (38). Diltiazem and verapamil act on a regulatory subunit of the voltage-dependent Ca^{2+} channel at concentrations 1–2 orders of magnitude below those used in this study (39). The liver is not throught to contain voltage-dependent Ca^{2+} channels but a Ca^{2+} gating-like effect has been observed in angiotensin-stimulated hepatocytes (40). The inhibitory effects of diltiazem and verapamil could conceivably reflect direct interaction of the receptor with another type of channel. The angiotensin receptor in renal tubular endothelium may be directly coupled to a non-voltage-sensitive Ca^{2+} channel (41). During desensitization, tissue response to angiotensin II is quantitatively and qualitatively altered, with possibly greater involvement of Ca^{2+} influx over mobilization of intracellular Ca^{2+} (31). This might be associated with interconversion of receptor subtypes as part of a regulatory mechanism. The ability to interfere pharmacologically with Ca^{2+} modulation of angiotensin receptor status may prove useful in further studies on this phenomenon.

In conclusion, the cation and nucleotide effects on the hepatic angiotensin receptor are similar to those in vascular tissue and are more complex than previously thought. This study indicates a biochemical basis for a number of pharmacological observations made on the angiotensin receptor, and the liver should provide a convenient model tissue in which to further study the role of cations and nucleotides in determining angiotensin receptor status.

References

1. McQueen, J., and P. F. Semple. Cation-dependent and cation-independent stages in regulation of the vascular angiotensin receptor after alteration of sodium balance in the rat. *Endocrinology* **121:** 1256–1264 (1987).
2. Douglas, J. G., G. Brown, and C. White. Influence of cations on kinetics of angiotensin II binding to adrenal, renal and smooth muscle receptors. *Hypertension (Dallas)* 4*(Suppl. III)*:III-79–84 (1982).
3. Wright, G. B., R. W. Alexander, L. S. Ekstein, and M. A. Gimbrone. Characterization of the rabbit ventricular myocardial receptor for angiotensin II. *Mol. Pharmacol.* **24:**213–221 (1983).
4. Baker, K. M., C. P. Campanile, G. J. Trachte, and M. J. Peach. Identification of the rabbit angiotensin II myocardial receptor. *Circ. Res.* **54:**286–293 (1984).
5. Douglas, J., G. Aguilera, T. Kondo, and K. Catt. Angiotensin II receptors and aldosterone production in rat adrenal glomerulosa cells. *Endocrinology* **102:**685–696 (1978).
6. Bennett, J. P., and S. H. Snyder. Regulation of receptor binding interactions of ^{125}I-angiotensin II and ^{125}I-[sarcosine^1,leucine^8]angiotensin II, an angiotensin antagonist, by sodium ion. *Eur. J. Pharmacol.* **67:** 1–10 (1980).
7. Brown, G. P., and J. G. Douglas. Angiotensin II binding sites on rat renal brush border membranes. *Endocrinology* **111:**1830–1836 (1982).
8. Childers, S. R., and S. H. Snyder. Differential regulation by guanine nucleotides of opiate agonist and antagonist receptor interaction. *J. Neurochem.* **34:**583–593 (1980).
9. Rosenberger, L. B., H. I. Yamamura, and W. R. Roeske. Cardiac muscarinic

receptor binding is regulated by sodium and guanyl nucleotides. *J. Biol. Chem.* **255**:820–823 (1980).
10. Chang, R. S. L., and S. H. Snyder. Histamine H_1 receptor binding sites in guinea pig brain membranes: regulation of agonist interactions by guanine nucleotides and cations. *J. Neurochem.* **34**:916–922 (1980).
11. Usdin, T. B., I. Creese, and S. H. Snyder. Regulation by cations of [^{3}H] spiroperidol binding associated with dopamine receptors of rat brain. *J. Neurochem.* **34**:669–676 (1980).
12. Schwarz, K. R., E. A. Carter, C. J. Homcy, and R. M. Graham. Agonist interaction of hepatic *alpha*$_1$ and *beta* adrenergic receptors: affinity state regulation by guanine nucleotides and temperature. *Biochemistry* **24**:7782–7788 (1986).
13. Gilman, A. G. Guanine nucleotide binding regulatory proteins and dual control of adenylate cyclase. *J. Clin. Invest.* **73**:1–4 (1984).
14. Crane, J. K., C. P. Campanile, and J. C. Garrison. The hepatic angiotensin receptor: effect of guanine nucleotides and interaction with cyclic AMP production. *J. Biol. Chem.* **57**:4959–4965 (1982).
15. Capponi, A. M., K. H. Van, and M. B. Vallotton. Differential effects of guanyl nucleotides on agonist and antagonist binding to rat adrenal and uterine angiotensin II receptors. *Eur. J. Pharmacol.* **114**:325–333 (1985).
16. Paiva, T. B., G. B. Mendes, and A. C. M. Paiva. Specific desensitisation (tachyphylaxis) of the guinea pig ileum to angiotensin II. *Am. J. Physiol.* **232**:H223–230 (1977).
17. Campanile, C. P., J. K. Crane, M. J. Peach, and J. C. Garrison. The hepatic angiotensin II receptor: characterization of the membrane binding site and correlation with physiological response in hepatocytes. *J. Biol. Chem.* **257**:4951–4958 (1982).
18. Capponi, A. M., and K. J. Catt. Solubilisation and characterization of adrenal and uterine angiotensin II receptors after photoaffinity labeling. *J. Biol. Chem.* **255**:12081–12086 (1980).
19. Sen, I., H. G. Bull, and R. L. Soffer. Isolation of angiotensin II binding protein from liver. *Proc. Natl. Acad. Sci. USA* **81**:1679–1683 (1984).
20. Alexander, R. W., T. A. Brock, M. A. Gimbrone, and S. E. Rittenhouse. Angiotensin increases inositol trisphosphate and calcium in vascular smooth muscle. *Hypertension (Dallas)* **7**:447–451 (1985).
21. Lynch, C. J., P. F. Blackmore, R. Charest, and J. H. Exton. The relationship between receptor binding capacity for norepinephrine, angiotensin II, and vasopressin and release of inositol trisphosphate, Ca^{2+} mobilization, and phosphorylase activation in rat liver. *Mol. Pharmacol.* **28**:93–99 (1985).
22. Cuatrecasas, P. Isolation of the insulin receptor of liver and fat cell membranes. *Proc. Natl. Acad. Sci. USA* **69**:318–322 (1972).
23. Kidwai, A. M., M. A. Radcliffe, E. Y. Lee, and E. E. Daniel. Isolation and properties of skeletal muscle plasma membrane. *Biochim. Biophys. Acta* **298**:593–607 (1973).
24. Kidwai, A. M., M. A. Radcliffe, and E. E. Daniel. Studies on smooth muscle plasma membrane: isolation and characterization of plasma membrane from rat myometrium. *Biochim. Biophys. Acta* **233**:538–549 (1971).
25. Cooperstein, S. J., and A. Lararow. A microspectrophotometric method for the determination of cytochrome oxidase. *J. Biol. Chem.* **189**:665–671 (1951).
26. McQueen, J., G. D. Murray, and P. F. Semple. Identification of the angiotensin II receptor in rat mesenteric artery. *Biochem. J.* **223**:659–671 (1984).
27. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand binding systems. *Anal. Biochem.* **107**:220–239 (1980).
28. Glossman, H., A. J. Baukal, and K. J. Catt. Properties of angiotensin II receptors in the bovine and rat adrenal cortex. *J. Biol. Chem.* **240**:825–834 (1974).
29. Chansel, D., N. Ardaillou, M. P. Nivez, and R. Ardaillou. Angiotensin II receptors in human isolated renal glomeruli. *J. Clin. Endocrinol. Metab.* **55**:961–966 (1982).
30. Moore, G. J., and Y. C. Kwok. Angiotensin receptors in resting smooth muscle cells are the low affinity sites observed in binding studies. *Life Sci.* **41**:505–511 (1987).
31. Maisiro, N., M. E. M. Oshiro, T. B. Paiva, and A. C. M. Paiva. Role of the two N-terminal residues of angiotensin II in the production of tachyphylaxis. *Eur. J. Pharmacol.* **87**:397–406 (1983).
32. Catt, K. J., M. C. Carson, W. P. Hausdorff, C. M. Leach-Harper, A. J. Baukal, G. Guillemette, T. Balla, and G. Aguilera. Angiotensin II receptors and mechanisms of action in adrenal glomerulosa cells. *J. Steroid. Biochem.* **27**:915–927 (1987).
33. De Lean, A., H. Ong, J. Gutkowska, P. W. Schiller, and N. McNicoll. Evidence for agonist-induced interaction of angiotensin receptor with a guanine nucleotide-binding protein in bovine adrenal zona glomerulosa. *Mol. Pharmacol.* **26**:498–508 (1984).
34. Moore, A., and P. A. Khairallah. Further studies on angiotensin tachyphylaxis. *J. Pharmacol. Exp. Ther.* **197**:575–581 (1976).
35. Ackerly, J. A., A. F. Moore, and M. J. Peach. Demonstration of different contractile mechanisms for angiotensin II and des-Asp^1-angiotensin II in rabbit aortic strips. *Proc. Natl. Acad. Sci. USA* **74**:5725–5728 (1977).
36. Freer, R. J., J. C. Sutherland, and A. R. Day. Synthesis and pharmacology of a noncompetitive antagonist of angiotensin-induced contractions of vascular smooth muscle. *Circ. Res.* **46**:720–725 (1980).
37. Aguilera, G., A. Schirar, A. Baukal, and K. J. Catt. Angiotensin II receptors: properties and regulation in adrenal glomerulosa membranes. *Circ. Res. Suppl.* **46**:I-118–I-127 (1980).
38. Stanfield, P. R. Voltage-dependent calcium channels of excitable membranes. *Br. Med. J.* **42**:359–367 (1986).
39. Glossmann, H., D. R. Ferry, F. Lubbecke, R. Mewes, and F. Hoffman. Identification of voltage operated calcium channels by binding studies: differentiation of subclasses of calcium antagonist drugs with ^{3}H-nimodipine radioligand binding. *J. Recept. Res.* **3**:177–190 (1983).
40. Woods, N. M., K. S. R. Cuthbertson, and P. H. Cobbold. Repetitive transient rises in cytoplasmic free calcium in hormone-stimulated hepatocytes. *Nature (Lond.)* **319**:600–602 (1986).
41. Welsh, C., G. Dubyak, and J. G. Douglas. Relationship between phospholipase C activation and prostaglandin E_2 and cyclic adenosine monophosphate production in rabbit tubular endothelial cells. *J. Clin. Invest.* **81**:710–719 (1988).

Send reprint requests to: Dr. P. F. Semple, MRC Blood Pressure Unit, Western Infirmary, Glasgow G11 6NT, United Kingdom.