The Hepatic Angiotensin II Receptor

I. CHARACTERIZATION OF THE MEMBRANE-BINDING SITE AND CORRELATION WITH PHYSIOLOGICAL RESPONSE IN HEPATOCYTES

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The angiotensin II binding sites on hepatic plasma membranes were characterized using the 125I-radiolabeled hormone and a partially purified preparation of rat liver membranes. Equilibrium binding studies carried out under optimal conditions yielded a curvilinear Scatchard plot. The nontransformed data were evaluated by computer and optimally fit to a two-site model with a high affinity site \( K_d = 0.21 \pm 0.06 \) nM and \( N = 229 \pm 72 \) fmol/mg of protein and a low affinity site \( K_d = 2.9 \pm 0.5 \) nM and \( N = 1820 \pm 185 \) fmol/mg of protein. The angiotensin antagonist, \(^{26} \text{Sarcosine}^8\text{Angiotensin II}^9\) (saralasin) bound to only one class of sites, corresponding to the low affinity high capacity site for angiotensin II with a \( K_d = 3.3 \pm 0.8 \) nM and \( N = 1780 \pm 171 \) fmol/mg of protein. The divalent cations \( \text{Ca}^{2+} \), \( \text{Mg}^{2+} \), and \( \text{Mn}^{2+} \) stimulated binding of angiotensin II greater than 9-fold, with half-maximal stimulation of binding occurring at 0.6-0.9 mM for the three ions. In contrast, saralasin binding was relatively unaffected by divalent cation concentrations between 0-2.0 mM and was inhibited at divalent ion concentrations above 2.0 mM. The high affinity binding site for angiotensin II was undetectable in the presence of 2 mM EDTA and progressively increased to 398 \pm 213 \) fmol/mg of protein as the level of free \( \text{Mg}^{2+} \) was raised to 10 mM. The \( K_d \) of the high affinity site was not affected by changes in \( \text{Mg}^{2+} \) concentration. The affinity and number of low affinity binding sites increased as the \( \text{Mg}^{2+} \) concentration was raised. In the absence of \( \text{Mg}^{2+} \), the low affinity site had a \( K_d = 13.4 \pm 4.4 \) nM and \( N = 579 \pm 139 \) fmol/mg of protein while at a \( \text{Mg}^{2+} \) concentration of 10.0 mM the \( K_d = 4.2 \pm 2.4 \) nM and \( N = 1500 \pm 312 \) fmol/mg of protein. A positive correlation was established between the ability of angiotensin analogues to stimulate phosphorlyase activity in intact hepatocytes and the analogues' potency in competing for angiotensin II binding sites in liver membranes. Together these data fulfill the criteria for positive identification of a hormone receptor including saturability, reversibility, specificity, and correlation with biological activity. These results provide strong evidence that this membrane-binding site is the hormone receptor which mediates the metabolic effects of angiotensin II in the liver.

Although the liver is not usually considered a target tissue for angiotensin II, this organ exhibits a number of responses to AII including stimulation of glycogenolysis (1, 2), stimulation of gluconeogenesis (3, 4), inhibition of fatty acid synthesis (5, 6), and stimulation of renin substrate production (7). In addition, preliminary studies have reported a hepatic binding site for angiotensin II (8, 9).

Physiologically, angiotensin II mimics the action of glucagon on carbohydrate metabolism in the liver. Glucagon acts in liver via the well understood mechanisms involving adenylate cyclase (10), cyclic AMP (11), and the cyclic AMP-dependent protein kinase (4, 12, 13). In contrast, relatively little is known about the mechanism of action of angiotensin II, although it appears to act through a \( \text{Ca}^{2+} \)-requiring cyclic AMP-independent pathway. The available data suggest that a possible sequence of angiotensin II action might be: binding of the hormone to a membrane receptor (8, 9); stimulation of phosphatidylinositol breakdown (14, 15); elevation of cytoplasmic \( \text{Ca}^{2+} \) levels (16, 17); activation of one or more \( \text{Ca}^{2+} \)-dependent protein kinases which phosphorylate the regulatory enzymes of carbohydrate metabolism (4).

Prior to investigating the biochemical events involved in the hepatic actions of angiotensin II, a binding assay for rat liver angiotensin II receptors was developed. The binding sites demonstrate high affinity, specificity, saturability, reversibility, appropriate kinetics, and correlation of binding with a biological effect. These characteristics suggest that the binding sites possess the properties expected of a hormone receptor and that these receptors mediate the metabolic effects of angiotensin II in the liver.

EXPERIMENTAL PROCEDURES

Materials

Angiotensin Analogues and Other Hormones—Asp',Ile'-AII and Ile'-AII were obtained from Beckman Instruments, Bachem Inc., or United States Biochemical Corp.; Sar',Ile'-AII was from Beckman Instruments; Sar',Ala'-AII and Sar',Leu'-AII were from Vega-Fox Biochemicals; Sar',Gly'-AII was from Peninsula Laboratories, Inc.; Asp',Ile'-AII was from Sigma Chemical Co. The following compounds were generously donated: des-Asp',Ile'-AII by Dr. G. Marshall, Washington University, St. Louis, MO; Asp'-α-aminobutyric acid-AII by Dr. M. Khosla, Cleveland Clinic; Sar'-Ile'-AII by Dr. A. Chiu, University of Oklahoma, Oklahoma City, OK; glucagon by Dr. Mary Root, Eli Lilly Co., Sar'-S-Me-Cys'-AII by Dr. R. Freer, Richmond VA.

Other Reagents—β-Aspartyl naphthylamide was purchased from Sigma Chemical Co. The compounds were generously donated: des-Asp',Ile'-AII by Dr. G. Marshall, Washington University, St. Louis, MO; Asp'-α-aminobutyric acid-AII by Dr. M. Khosla, Cleveland Clinic; Sar'-Ile'-AII by Dr. A. Chiu, University of Oklahoma, Oklahoma City, OK; glucagon by Dr. Mary Root, Eli Lilly Co., Sar'-S-Me-Cys'-AII by Dr. R. Freer, Richmond VA.

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The abbreviations used are: AII, Asp',Ile'-angiotensin II; AIII, des-Asp'-angiotensin II; AI, Ile'-angiotensin I; Sar, sarcosine; saralasin, Sar',Ala'-angiotensin II; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethane, BSA, bovine serum albumin.
**Methods**

**Preparation of Rat Liver Plasma Membranes**—Rat liver membranes were prepared from 200- to 300-g male Wistar rats according to the protocol (through step 10) of Pohl (18) which is based on the original procedure of Neville (19). However, 10 mM Tris (pH 7.4) was substituted for 1 mM NaHCO3, and dithiothreitol (1 or 5 mM) or mercaptoethanol (0.1%) was added to preserve adenylate cyclase activity. For experiments testing the effects of ions on binding of angiotensin to its receptor, membranes were prepared in a buffer consisting of 5 mM EDTA in 10 mM Tris, pH 7.4. Membranes were stored in liquid N2 for up to 4 months at a concentration of 5-6 mg of protein/ml without loss of binding activity.

Electron micrographs of the liver plasma membranes revealed that the preparation was composed of long continuous sheets of membrane folded back on itself giving an overall vesicular appearance (not shown); the preparation was prepared in a buffer containing the basal activity of the marker enzyme adenylate cyclase was 9 pmol of cAMP/mg of protein/min which increased to 214 pmol of cAMP/mg of protein/min in the presence of 10-7 M glucagon and 10-4 M GTP. These adenylate cyclase activities are in the range of those previously reported for this membrane preparation (20). Adenylate cyclase was assayed as described in an accompanying article (21).

**Iodination of Peptides and Determination of Specific Activity and Purity**—Angiotensin II and saralasin were iodinated using the chloramine-T procedure of Hunter and Greenwood (22) as modified by Freedlander and Goodfriend (23). The products of the iodination reaction were separated using a DEAE-Sephadex A-25 column, and the monooiodinated peptide fraction was collected and stored in 200-µl aliquots in 0.8 m glycine buffer, pH 7.5, at -75 °C. Aliquots of the 125I-labeled peptide were thawed immediately before use in radioligand-binding studies and never reused. The purity of the iodinated peptides was evaluated by thin layer chromatography on Eastman plastic-backed cellulose plates developed in a-butyl alcohol:acetic acid:methanol:water (15:3:10:12). About 95-100% of both the 125I-AII prepared in this laboratory and that purchased from New England Nuclear co-migrated with the angiotensin standard in the chromatography system. The purity of the 125I-saralasin was judged to be 85-90% by the same criteria. The specific activity of the 125I-saralasin was 926 Ci/mmol as determined by the self-displacement method of Berson et al. (24) using a rabbit antibody to saralasin. The 125I-AII used for saturation binding isotherms was obtained from New England Nuclear and used according to its stated specific activity. While the specific activity of the angiotensin standards separated in this laboratory was not measured, based on its binding activity it appeared to have a specific activity similar to that obtained from New England Nuclear.

**Binding Experiments**—The buffer originally chosen for binding experiments (20 mM HEPES, 150 mM NaCl, 0.2% crystalline BSA, pH 7.4) was similar to the conditions employed by Glossmann et al. (25). However, experiments designed to optimize binding conditions resulted in the addition of liver membranes (5.0 mg of protein/ml) to the incubation tube at varying times, filtered, and rinsed twice with 2 ml of Tris buffer. The filters were counted in a Beckman Biogamma Counter with an efficiency of 90-95%. Experimental results are expressed as saturable binding, which was defined as that portion of total binding able to be displaced by an excess concentration (10-15 M) of unlabeled ligand. Saturable binding constituted 90-95% of total binding (125I-angiotensin concentrations of 0.1-0.5 nM).

**Hormone Metabolism Studies**—Metabolism of angiotensin II by rat liver plasma membranes was examined by incubating 0.1-0.3 nM 125I-labeled ligand with membranes at a concentration of 0.5 mg of protein/ml for 60 min at 0, 12, 22, and 37 °C. Bound and free ligand were separated by centrifugation. The bound hormone was solubilized by adding 100 µl of 50% glacial acetic acid to the pellet followed by incubation in a boiling water bath for 5 min. The free and bound hormone, along with radiolabeled angiotensin standards, were spotted on an Eastman plastic-backed cellulose thin layer chromatography plate and developed in n-butyl alcohol:acetic acid:pyridine:water (15:3:10:12). Plates were scanned with a Berthold thin layer radio scanner as well as cut up into strips and counted in a Beckman Biogamma Counter. High voltage paper electrophoresis was also used to monitor metabolism of bound and free 125I-angiotensin II using a solvent system of water:acetic acid:pyridine (27:21:1) at pH 4.2.

**Analysis of Binding Data**—The dissociation constant and number of binding sites for all saturation binding isotherms were determined using a nonlinear least squares curve fitting program (26) to fit the data to a one- or two-site model based on the equation

\[ B = N \left( L/(L + K_d) \right) + N (L/(L + K_{d2})) \]

where \( B \) = ligand bound, \( L \) = free ligand concentration, \( N \) = concentration of binding sites, and \( K_d \) = dissociation constant of the binding site. For a one-site model the second term on the right side of the equation is dropped. The program was executed on a Tektronix 4006 graphics terminal linked to a Control Data Corporation Cyber 720 computer. Goodness of fit was judged by the variance obtained from a given curve fit. A graphic display of the line drawn through the experimental points allowed a visual confirmation of the computer-calculated fit. The computer program determined the parameters for the one- or two-site equation directly from the saturation binding data. However, the linearity or deviation from linearity of a Scatchard analysis more dramatically depicts the presence of one or more binding sites. For this reason, some of the data presented under "Results" have been transformed to a Scatchard plot.

**Preparation of Hepatocytes and Measurement of Enzyme Activities**—Isolated liver cells were prepared from 300- to 350-g fasted male Wistar rats by published methods (27). The hepatocytes were resuspended in Krebs-Ringer bicarbonate buffer to a concentration of about 20 mg of protein/ml and gassed with 95% O2, 5% CO2. One to two milliliters of cell suspension were preincubated for 30 min with 16 mM l-lactate and 4 mM pyruvate under 95% O2, 5% CO2 at 37 °C. Following the preincubation, cells were stimulated with the various angiotensin analogues for 2 min prior to sampling for the phosphorylase assay and for 5 min prior to sampling for the glycogen synthase assay. These sample times have been determined to give optimal responses (4). Cell extracts for the assay of phosphorylase and glycogen synthase were prepared according to published methods (27). Flowthrough activity was assayed according to the method of Stalmans and Hers (28) adapted to the filter paper technique, and glycogen synthase was assayed as described by Thomas et al. (29).

**RESULTS**

**Establishment of Optimal Conditions for Binding**—Preliminary experiments were carried out to provide information on the kinetics, ion, and temperature dependence and stability of binding. These experiments employed a buffer containing 100 mM NaCl, 0.2% crystalline BSA, and 20 mM Tris, following the conditions of Glossmann et al. (25). The rate of association of angiotensin II with rat liver membranes was directly proportional to the temperature of incubation. Maximal binding occurred in 25 min at 22 °C compared to 90 min at 4 °C. The
stability of binding was inversely proportional to temperature. At 22 °C binding began to decrease immediately from maximal levels, while at 4 °C maximal binding was maintained for more than 30 min. Binding kinetics at 12 °C offered a compromise, with maximal levels being reached within 50–60 min and maintained for an additional 20 to 30 min.

Degradation of angiotensin II by the hepatic membranes could explain the short-lived steady state binding observed at 22 °C. However, a number of agents, including dithiothreitol, β-aspartyl naphthalamide, bacitracin, and the oxidized β-chain of insulin, which have been shown to inhibit the degradation of angiotensin and other peptides (25, 30–32), failed to prevent the rapid loss of membrane-bound angiotensin II at 22 °C. Direct measurement of [125I]-angiotensin II degradation by both ascending chromatography and high voltage electrophoresis indicated that there was negligible metabolism of bound [125I]-AII after a 1-h incubation with membranes at 4, 12, 22, or 37 °C. Furthermore, there was no detectable metabolism of free [125I]-AII following a 1-h incubation with membranes at 4, 12, or 22 °C. Even at 37 °C, 90% of the free radioactivity was co-precipitated with radiolabeled hormone standard. These data demonstrated that there was minimal degradation of the ligand and suggested that the angiotensin binding site itself might be labile. In support of this hypothesis, preincubation of membranes at 4, 12, 22, or 37 °C demonstrated a temperature-dependent decrease in [125I]-AII binding. The greatest decrease in binding (70–100%) occurred after preincubation of the membranes for 75 min at 37 °C.

Attempts to stabilize the AII binding activity of rat liver membranes revealed that 10 mM Mg2+ prevented the time-dependent loss of binding when this ion was present during both the preincubation of membranes and the equilibration with the ligand. At all preincubation temperatures except 37 °C, Mg2+ prevented the loss of binding capacity. After preincubation at 37 °C, binding in the presence of Mg2+ was 50% of control values. Furthermore, the presence of Mg2+ in the incubation mixture was able to restore the binding activity lost after preincubating membranes in a Mg2+-free buffer at 4, 12, 22, or 37 °C. Consequently, in most studies incubations were performed in 100 mM NaCl, 10 mM MgCl2, 20 mM Tris, and 0.2% crystalline BSA, at 12 °C. However, in experiments designed to study ion dependence the buffer contained only Tris and BSA.

**Determination of Binding Parameters for Angiotensin II and Saralasin—**Fig. 1 shows the dose-dependent binding of [125I]-angiotensin II to liver membranes. The relatively flat binding isotherm spans more than three orders of magnitude (Fig. 1A), with a threshold concentration of 5 × 10^-12 M AII and saturation of sites occurring at a concentration of about 2 × 10^-10 M AII. As suggested by the shallow dose response curve, when the data were transformed according to Scatchard (33) an upwardly concave curvilinear plot was obtained. This result is indicative of either more than one class of binding sites or negative cooperativity. Computer analysis of the saturation binding data of Fig. 1A yielded an optimal fit to a two-site model with a high affinity site K<sub>H</sub> = 2.05 ± 0.4 nM and number of sites N<sub>H</sub> = 216 ± 32 fmol/mg of protein and a low affinity site K<sub>L</sub> = 3.2 ± 0.4 nM and number of sites N<sub>L</sub> = 995 ± 88 fmol/mg of protein. The average binding parameters for 10 such experiments, with each point determined in triplicate, yielded the following values: K<sub>H</sub> = 0.21 ± 0.06 nM, N<sub>H</sub> = 229 ± 72 fmol/mg of protein, K<sub>L</sub> = 2.9 ± 0.5 nM, N<sub>L</sub> = 1826 ± 185 fmol/mg of protein. Six different membrane preparations were used in this pool of 10 experiments.

The affinity of a hormone-receptor interaction can also be obtained from the ratio of the association and dissociation rates for hormone binding. Rigorous kinetic analysis of this type is very difficult.
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this end, $^{125}$I-saralasin, an antagonist radioligand, was prepared. Fig. 2 represents a typical saturation isotherm for $^{125}$I-saralasin binding to rat liver membranes. The data, expressed as a Scatchard plot, yielded an apparent straight line indicative of one class of binding sites. This finding was confirmed by computer analysis of the data which yielded a best fit to a single class of sites with $K_d = 3.9 \pm 1.3 \text{ nM}$ and number of sites $N = 1978 \pm 529 \text{ fmol/mg of protein}$. The average of four experiments, using two membrane preparations with each point performed in triplicate, gave a $K_d = 3.3 \pm 0.8 \text{ nM}$ and $N = 1780 \pm 171 \text{ fmol/mg of protein}$. The binding parameters for saralasin correspond well to the low affinity high capacity angiotensin II binding site.

Effects of Cations on Agonist and Antagonist Binding—In a number of other systems ions have been shown to enhance or depress hormone binding as well as have differential effects on agonists and antagonists (36-39). Based on these findings it was of interest to investigate the ion dependence of $^{125}$I-angiotensin II and $^{125}$I-saralasin binding to liver membranes. These experiments were performed with membranes prepared and washed several times in Tris buffer containing 5 mM EDTA to minimize their divalent cation concentration. As shown in the top half of Fig. 3, the divalent cations, Mg$^{2+}$, Ca$^{2+}$, and Mn$^{2+}$, stimulated AII binding up to 7-fold over control levels. The $E_{D50}$ for this divalent cation potentiation of agonist binding ranged from 0.6 mM for Mg$^{2+}$ to 0.9 mM for Mn$^{2+}$. At divalent cation concentrations above 20 mM, binding gradually decreased but still remained well above control

![Graph](https://via.placeholder.com/150)

Fig. 2. Scatchard plot of $^{125}$I-saralasin binding to liver membranes. Varying concentrations of $^{125}$I-saralasin were incubated with membranes (0.5 mg/ml) for 60 min at 12 °C. Bound and free ligand were separated as described under "Methods." The line through the points was generated using a least squares linear regression program. Computer analysis of the data yielded a one-site fit with a $K_d = 2.0 \text{ nM}$ and $N = 1978 \pm 529 \text{ fmol/mg of protein}$. Points represent saturable binding and are the mean of three observations ± S.E. This figure is representative of four such experiments performed utilizing two separate membrane preparations.

![Graph](https://via.placeholder.com/150)

Fig. 3. Ion dependence of angiotensin II and saralasin binding to liver membranes. $^{125}$I-Angiotensin II (~0.3 nM, top) or $^{125}$I-saralasin (~0.4 nM, bottom) was incubated with liver membranes (0.5 mg/ml) in the presence of varying concentrations of Ca$^{2+}$ (■ ■), Mg$^{2+}$ (○ ○), Mn$^{2+}$ (▲ ▲), Na$^{+}$ (□ □), or K$^{+}$ (⊗ ⊗). After 60 min at 12 °C saturable binding was determined as described under "Methods." Data are expressed as "% control/100" which is equivalent to "-fold increase over control," control being 20 mM Tris, 0.2% crystalline BSA, pH 7.5, with no added ions. Data points represent pooled results from one, two, or three experiments, each one performed in triplicate. Standard errors of the mean were omitted for clarity's sake but were ≤8% in all cases. The inset in the bottom is a replot of the saralasin data with an expanded ordinate.
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Angiotensin II saturation binding curves were carried out in the presence of 2.0 mM EDTA with varying concentrations of free Mg$^{2+}$. $[^{3}H]$ Angiotensin II and rat liver membranes (0.5 mg of protein/ml) were incubated for 60-70 min at 12°C, and bound and free ligand was separated by filtration as described under "Methods." Binding parameters were also determined in the presence of Krebs-Ringer buffer with the bicarbonate being replaced by Tris (KR Tris). Each point was determined in triplicate with a control for nonsaturable binding. The data were computer fit to a one- or two-site model as described under "Methods." Results in this table are presented as the mean of 3-5 experiments ± S.E. using three separate membrane preparations.

### TABLE I

**Angiotensin II binding parameters as a function of Mg$^{2+}$ concentration**

| [Mg$^{2+}$] (mM) | N$_1$ (fmol/mg protein) | K$_{o1}$ (nM) | N$_2$ (fmol/mg protein) | K$_{o2}$ (nM) |
|-----------------|------------------------|--------------|------------------------|--------------|
| 0               | 36 ± 14                | 0.44 ± 0.28  | 280 ± 183              | 16.0 ± 14.0  |
| 0.01            | 243 ± 61               | 0.78 ± 0.17  | 797 ± 461              | 16.5 ± 2.0   |
| 0.5             | 388 ± 213$^b$          | 0.49 ± 0.21  | 1500 ± 312            | 4.2 ± 2.4    |
| 5.0             | 153 ± 39               | 0.74 ± 0.3   | 1787 ± 751            | 8.3 ± 0.7    |

$^a$ Not significantly different (p > 0.05) from value obtained at 0.01 mM Mg$^{2+}$.

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**Statistical significance was evaluated using the unpaired Student’s t test.**

The bottom half of Fig. 3 shows that in contrast to agonist binding, divalent cations between a concentration of 0.5 and 5.0 mM only slightly increased saralasin binding. However, at concentrations above 5.0 mM there was a marked decrease in saralasin binding with nearly complete inhibition at 100 mM divalent ion. The monovalent cations, sodium and potassium, did not display any potentiation of saralasin binding but at the higher concentrations also caused significant inhibition.

Since ions were found to have such a pronounced effect on angiotensin II binding, it was of interest to examine the effect of Mg$^{2+}$ on the concentration or affinity of the two AI1 binding sites. Using EDTA-prepared membranes, the addition of 2 mM EDTA to the incubation mixture lowered AI1 binding by 25-75% depending on the particular membrane preparation. The EDTA in the assay also slightly lowered the half-maximal concentration of free Mg$^{2+}$ needed for stimulation of AI1 binding from 0.6 to 0.5 mM. Angiotensin II saturation binding curves were generated in the presence of threshold (0.01 mM), half-maximal (0.5 mM), or maximal (10.0 mM) concentrations of free Mg$^{2+}$ as determined in pilot experiments. The data were fit by computer to a one- or two-site model as described under "Methods." In the presence of 2 mM EDTA without added Mg$^{2+}$ no binding of $[^{3}H]$AI1 to the high affinity low capacity site was observed (Table I). Binding data were best fit to a single class of low affinity sites with a K$_d$ = 13.4 nM.

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**Fig. 4. Hormone specificity of the rat liver angiotensin II binding site.** $[^{3}H]$ Angiotensin II (0.3 nM) was incubated with liver membranes (0.5 mg of protein/ml) in the presence of varying amounts of unlabeled angiotensin analogues or other hormones. After incubation at 12°C for 60 min, bound and free ligands were separated by filtration as described under "Methods." Data are corrected for nonsaturable binding and are plotted as % control binding. Control being the addition of buffer instead of hormone analogue. Data points represent the mean of pooled data from two separate experiments, each performed in triplicate. Standards errors of the mean were omitted for clarity’s sake but were ≤6% in all cases. Sar, sarcosine; Iso, isoproterenol.
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Table II

Binding potency (Kd) versus phosphorylase activation (Kact) of angiotensin analogues

Kd values were obtained from competition curves in which 125I-angiotensin II (0.3 nM) was incubated with liver membranes (0.5 mg of protein/ml) in the presence of varying concentrations of the unlabeled analogues listed in this table. Saturable binding was determined as described under "Methods." ID50 values were obtained graphically from the curve described by the data points for each analogue. The competition curve for the Ang II was also computer generated with a nonlinear curve fitting program and agreed well with the graphically determined curve. Kd values were obtained from the equation Kd = ID50 (1 - f) where f equals fractional occupancy of the binding site by 125I-AII. Kd values were determined graphically from the data of Fig. 5. Kd is defined as the concentration of agonist needed to provide 50% of maximal phosphorylase activation.

| Hormone          | Kd   | Kact |
|------------------|------|------|
| Angiotensin II   | 4.1  | 1.6  |
| Angiotensin III  | 11.8 | 4.6  |
| Angiotensin I    | 1455 | 316  |
| des-Asp'-angiotensin I | 18,182 | 73,500 |

* Obtained by extrapolation to 50% inhibition of binding or 50% activation of phosphorylase activity.

Specificity for Hormone Binding and Correlation of Binding and Biological Potency—Fig. 4 demonstrates the specificity requirements of the angiotensin molecule for interaction with its binding site in rat liver membranes. Substitution of sarcosine for aspartic acid in position 1 and several aliphatic amino acids for phenylalanine in position 8 converts the native hormone into a competitive antagonist in most angiotensin target tissues (42, 43). In liver membranes this class of analogues displayed an affinity for the Ang II binding site, which was equipotent with the native hormone, Ile8-AII. Sar1-S-Me-Cys3, Ang III, a noncompetitive angiotensin antagonist in smooth muscle (44), was 65% as potent as Ang II in displacing 125I-AII from liver membranes (data not shown). Des-Asp1, Ile8-AIII, the angiotensin III antagonist, had a Kd seven times greater than that of Ile8-AII. Asn1-α-aminobutyrate8-AII, a potent agonist for stimulation of water transport in rat jejunum, had an ID50 greater than 10 μM for inhibition of 125I-AII binding to liver membranes. Glucagon, isoproterenol, and vasopressin, other hormones which regulate carbohydrate metabolism in liver, did not compete for angiotensin binding sites in liver membranes.

The potency of a series of angiotensin agonists in displacing 125I-AII bound to liver membranes was compared with their ability to stimulate glycogen phosphorylase activity in intact rat hepatocytes (Fig. 5). Phosphorylase activation was chosen for this comparison because the response to angiotensin II is large, rapid, and closely correlated with receptor occupancy (45, 46).

Table II compares the Kd and Kact values obtained from the curves shown in Fig. 5. Although the Kd and Kact values were

![Fig. 5. Comparison of stimulation of phosphorylase activity by angiotensin (Ang) agonists with their ability to displace the binding of 125I-Ang II. Top, isolated hepatocytes were preincubated for 30 min, stimulated with the indicated angiotensin analogues for 2 min, and then sampled and assayed for phosphorylase activity. The data were normalized to % of control. Basal phosphorylase activity was 0.25 ± 0.02 μmol of glucose-1-P/mg of protein/15 min (N = 12). All angiotensin analogues except des-Asp1-Ang I caused a maximal stimulation of phosphorylase equal to 0.70 ± 0.06 μmol of glucose-1-P/mg of protein/15 min (+285%). Each point represents the mean from 2-3 experiments, with each assay performed in duplicate. Bottom, 125I-Ang II (0.3 nM) was incubated with liver membranes (0.5 mg/ml) in the presence of varying concentrations of unlabeled AII, AIII, Al, or des-Asp1-AI. After incubation at 12 °C for 60 min, bound and free ligand were separated by filtration as described under "Methods." Data are corrected for nonsaturable binding and are plotted as % of control binding, control being the addition of buffer instead of hormone analogue. Data points represent the mean of pooled data from two separate experiments, each one performed in triplicate. Standard errors were omitted for clarity’s sake but were ≤6% in all cases.

In gut (40) and kidney cortex (41) sar1, aliphatic amino acid8-angiotensin IIs are full agonists.
not identical for a given analogue, the relative potencies among the angiotensins for binding and biological activity were very similar. The $K_d$ values for binding of angiotensin analogues were 3- to 5-fold greater than the respective $K_m$ values for glycinogen phosphorylase activation. However, the reverse was true for des-Asp$^1$-AI, the least potent agonist tested. Stimulation of phosphorylase activity by angiotensin I was not affected by captopril (data not shown), which indicated that the activity of the decapptide was not dependent upon its metabolism to AII by angiotensin-converting enzyme. In addition to the close correspondence with phosphorylase activation there was also a good correlation between the $K_d$ of AII binding (4.1 nM) and its ability to inhibit glycogen synthase activity ($K_{act} = 1.7$ nM) in intact hepatocytes (data not shown).

**DISCUSSION**

This study characterizes the rat hepatic angiotensin II binding site and provides evidence indicating this site to be the receptor which mediates the metabolic effect of angiotensin II on carbohydrate metabolism in the liver. The following criteria, necessary for positive identification of a hormone receptor, have been satisfied: affinity commensurate with physiologic concentrations of the hormone (Fig. 1), saturability (Fig. 1), reversibility (21), specificity (Figs. 4 and 5), and most importantly, correlation of binding and biological effect of hormone analogues (Fig. 5). The binding affinities described here for liver are very similar to those seen by others for angiotensin II binding to rat adrenal cortex homogenate in which high and low affinities of 0.2 nM and 6.3 nM, respectively, were reported (25). Our data, which show two classes of angiotensin II binding sites, are similar to those reported in bovine (25), dog (47), and rat (48) adrenal cortical homogenate. In contrast, only one class of binding sites for angiotensin II was observed in rat mesenteric artery (37) and dog uterine homogenate (47). However, since the affinity and concentration of the two sites in liver membranes appear to be critically dependent on ionic and other experimental conditions (text, Fig. 3, and Table I), it is difficult to compare hepatic angiotensin II receptor which mediates the metabolic effect of angiotensin II to hepatic membranes indicating that the rat mesenteric artery homogenate, though the magnitude of binding potentiation in the mesenteric artery was much less than what we observed in rat liver plasma membranes.

There is a striking resemblance between the effects of cations on angiotensin binding presented here and the data reported by Williams et al. (38) for $\beta$-adrenergic agonist and prostaglandin binding to frog erythrocyte membranes. These authors demonstrated that the divalent cations Mg$^{2+}$, Mn$^{2+}$, and Ca$^{2+}$ caused a marked potentiation of AII binding and cation concentrations important not only in the mechanism of action of angiotensin II in the liver cytosol but also in regulating the initial hormone-receptor interaction. Sodium caused a comparatively small (3-fold) potentiation of AII binding while potassium had no effect. This profile of ion dependence of AII binding is very similar to that reported by Gunther et al. (37) for rat mesenteric artery homogenate, though the magnitude of binding potentiation in the mesenteric artery was much less than what we observed in rat liver plasma membranes.

In all of these systems the following order of potency was consistent with our results the stimulation of $\beta$-adrenergic agonist binding was decreased at higher divalent cation concentration. More importantly, the potentiating effects of divalent cations on both $\beta$-adrenergic and angiotensin binding were agonist-specific. For example, magnesium did not increase the affinity of propranolol binding to erythrocyte membranes (38) nor that of saralasin binding to liver membranes. Similarly, Bird and Maguire (39) reported that the potentiation effect of Mg$^{2+}$ on the binding of $\beta$-adrenergic ligands to membranes from S49 lymphoma cells was agonist-specific. One distinguishing feature of the S49 membranes was that the effects of ions were limited to Mg$^{2+}$.

Williams et al. (38) postulated that Mg$^{2+}$ ion did not directly affect the interaction of ligand with its receptor but rather acted on adenylate cyclase or the guanine nucleotide regulatory protein to promote the formation of a high affinity receptor state. Perhaps more revealing from a mechanistic point of view are the data of Bird and Maguire directly implicating the involvement of the guanine nucleotide regulatory protein in the action of Mg$^{2+}$ (39). Their work demonstrated a lack of Mg$^{2+}$ stimulation of $\beta$-adrenergic agonist binding in genetic variants of S49 lymphoma cells in which the guanine nucleotide regulatory protein is absent or functionally inactive. The parallels between the data obtained with $\beta$-adrenergic agents and that presented in the study reported here suggest a similarity between angiotensin II and adenylate cyclase-linked hormones. This hypothesis is investigated further in the accompanying article.

Our data differ from that of Bird and Maguire (39) and Williams et al. (38) in that while we observed a Mg$^{2+}$-dependent increase in both receptor affinity and number, these investigators demonstrated an effect on receptor affinity only. It is not clear how Mg$^{2+}$ is able to cause a change in the apparent number of hepatic angiotensin receptors. However, the differences between our results and those obtained with
The glycemic of insulin deficiency. With the characterization of the hepatic angiotensin receptor presented here, it will be anticipated that this approach will provide more information defining the physiological role of angiotensin in the liver.

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