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Characterization of the Rabbit Ventricular Myocardial Receptor for Angiotensin II

Evidence for Two Sites of Different Affinities and Specificities

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SUMMARY

Angiotensin II binding sites in a rabbit ventricular myocardial particulate fraction were identified and characterized with the radioligand ^{125}I-angiotensin II. The order of potency in competing with ^{125}I-angiotensin II for these sites was similar to that observed in physiological studies. Computer-assisted analysis of the competition of binding sites for 0.3 nM ^{125}I-angiotensin II by unlabeled angiotensin II (3×10^{-11} M to 1×10^{-5} M) demonstrated that optimal fitting of the competition curves was attained with a two-site model having one site of high affinity ($K_{A1} = 2.4 \pm 0.6 \times 10^9$ M^{-1}), low capacity ($N_1 = 7.8 \pm 0.8$ fmoles/mg of protein) and a second site low affinity ($K_{A2} = 9.6 \pm 0.6 \times 10^6$ M^{-1}) and high capacity ($N_2 = 219 \pm 128$ fmoles/mg of protein). Analysis of competition by Sar^1-Ile^8 angiotensin II for ^{125}I-angiotensin II binding sites indicated that the antagonist interacted with the first site with high affinity ($K_{A1} = 8 \times 10^9$ M^{-1}), but interacted minimally with the second site ($K_{A2} = 10^5$ M^{-1}). Monovalent cations (Na^+, K^+, Li^+, NH_4^+) were roughly equipotent in decreasing ^{125}I-angiotensin II binding by reducing the number of high-affinity sites ($N_1 = 2.6 \pm 0.7$ fmoles/mg of protein with 100 mM Na^+) without changing the affinity of either site or the number of low-affinity sites. The number of high-affinity sites was increased to 14.4 ± 1.5 fmoles/mg of protein by 5 mM Mg^{2+}. In the presence of divalent cations, nucleotides reduced binding of ^{125}I-angiotensin II with the potency order guanosyl-5′-yl-imidodiphosphate > GTP > GDP > ATP > GMP. Guanosyl-5′yl-imidodiphosphate significantly reduced the affinity of the high-affinity site ($K_{A1} = 1.0 \pm 0.2 \times 10^9$ M^{-1}) and perhaps of the low-affinity site ($K_{A2} = 1.0 \pm 2.2 \times 10^6$ M^{-1}). Computer-assisted assessment of dissociation of 0.3 nM ^{125}I-angiotensin II from rabbit myocardial membranes corroborated the equilibrium data: dissociation was biphasic ($k_{-1} = 0.19 \pm 0.2$ min^{-1} for a rapidly dissociating site, $k_{-1} = 2.5 \pm 2.1 \times 10^{-3}$ min^{-1} for a slowly dissociating site); 5 mM Mg^{2+} did not significantly change either dissociation rate; but guanosyl-5′-yl-imidodiphosphate significantly increased dissociation rates from both sites. Despite the indirect evidence that these angiotensin II receptors interact with guanine nucleotide regulatory proteins, angiotensin II (10^{-6} M) failed to influence adenylate cyclase activity. The physiological implications of the presence in ventricular myocardium of two distinct angiotensin II receptors and in particular the implications of a receptor-associated guanine nucleotide regulatory protein which does not couple to adenylate cyclase require further investigation.

INTRODUCTION

Angiotensin II, a potent stimulator of vasoconstriction and adrenal steroid production, also exerts positive cardiac inotropic effects (1–4). Pharmacological evidence suggests that angiotensin II enhances myocardial contractility both directly and indirectly. The peptide can act directly to enhance calcium influx (2, 5, 6) and to increase electrical coupling among myocytes (7), and indirectly to enhance the release of catecholamines from cardiac sympathetic nerve terminals (8).

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Angiotensin II receptors, which have been characterized by radioligand binding techniques in a wide variety of tissues, display considerable tissue-related heterogeneity (9, 10). This heterogeneity has been reflected in differences in the manner in which binding is affected by cations (11–19), in the heterogeneity of binding sites in a given tissue (20, 21), and in the coupling to adenylate cyclase (22–27). Early studies demonstrated radioligand binding to cardiac tissue (28), but more detailed characterization of this cardiac receptor has begun only recently (29–31). There is little information concerning the molecular mechanisms regulating the interaction of angiotensin II with its myocardial receptor.

As an approach to the problem of defining, at a molecular level, the physiology of the myocardial angiotensin II receptor, we have used ^{125}I-angiotensin II to characterize this receptor in rabbit heart, a tissue in which positive inotropic affects have been well demonstrated (3). The data indicate that there are two classes of binding sites, one of high affinity and low capacity, the other of low affinity and high capacity. Monovalent and divalent cations regulate the binding of angiotensin II only to the high-affinity site, but guanine nucleotides decrease the affinities of both sites for the peptide by accelerating the rates of dissociation. Despite the evidence that the receptor interacts with a membrane guanine nucleotide regulatory protein, no effect of angiotensin II on adenylate cyclase activity was discerned.

METHODS

Materials. ^{125}I-Angiotensin II (1200–1880 μCi/μg; New England Nuclear Corporation, Boston, Mass.) was reconstituted to a concentration of approximately 40 nM. Aliquots were flash-frozen (dry ice-acetone), stored at −20°, and were used only once after thawing. Purity was >98% by thin layer chromatography. Sar^1-Ala^8 angiotensin II was obtained from Peninsula Laboratories (San Carlos, Calif.), and Sar^1-Ile^8 angiotensin II was obtained from both Peninsula Laboratories and Beckman Instruments (Fullerton, Calif.). Bovine serum albumin (Pentex, Grade V) was obtained from Miles Laboratories (Elkhart, Ind.). All other chemicals were obtained from Sigma Chemical Company (St. Louis, Mo.). Cation additives were in the form of the chloride salts. Gpp(NH)p[2] was stored as an aqueous solution (0.02 M) at −20°. All other nucleotide solutions were prepared freshly for each assay. Angiotensin II analogues were stored as aqueous stock solutions (2×10^{-4} M) with 0.25% bovine serum albumin. Aliquots were thawed only once.

Tissue preparation for ^{125}I-angiotensin II binding assays. Rabbits were fed standard laboratory chow and had free access to tap water. For most assays, one male New Zealand White rabbit (2.5–3.0 kg) was killed by injecton of 0.25 ml of a veterinary euthanasia agent (T-61, National Laboratories, Baltimore, Md.). In three rabbits killed by blows to the head, the results of the binding assays did not differ from the results of assays on heart tissue obtained from injected rabbits. The heart was removed while still beating and was placed in cold phosphate-buffered saline. The atria, epicardial coronary arteries, great arteries, and fat were trimmed from the ventricles, which then were minced with a scalpel. The tissue was homogenized in 80 ml of 0.25 M sucrose (Brinkmann Polytron, three times, 10 sec, setting 10). The homogenate was centrigued at 4° for 10 min at 1,500 × *g*. The supernatant was carefully pipetted off and centrifuged at 4° for 10 min at 48,000 × *g*. The resulting supernatant was discarded, and the pellet was suspended in 50 mM Tris buffer (pH 7.50) and centrifuged for 5 min at 48,000 × *g*. The pellet was resuspended in 40 ml of 50 mM Tris (pH 7.5) containing 5 mM Na_2 EDTA and 0.1 mM PMSF (15). The tissue suspension was then "preincubated" in an Ehrlenmeyer flask for 30 min at 30° in a gyratory metabolic shaker. The preincubated tissue was centrifuged at 4° for 5 min at 48,000 × *g*. The pellet was reconstituted in 50 mM Tris buffer (pH 7.50 at 25°), usually to a volume of 10 ml (a protein concentration typically between 2.5 and 3.5 mg/ml). The resuspended tissue homogenate appeared to contain membranes with no nuclei or intact cells when viewed by phase-contrast microscopy.

Binding assay. Most binding assays were performed in polystyrene tubes (12 × 75 mm) (Sarstedt 55476) by incubating 0.10-ml aliquots of tissue homogenate (250–350 μg of protein) with 0.10 ml of an "assay mix" such that each assay tube (0.20 ml) contained 50 mM Tris (pH 7.50 at 25°), 0.25% bovine serum albumin, 0.1 mM PMSF, 5 μg of bacitracin, and 0.3 nM ^{125}I-angiotensin II, as well as various additives (cations, nucleotides, or unlabeled angiotensin II analogues). Assay tubes were incubated at 25° for 60 min (sufficient time to attain steady-state binding). To terminate the assay, 4.5 ml of ice-cold 0.9% NaCl were added to each tube. Bound and free radioactivity were separated by immediate filtration through glass-fiber filters (Whatman GF/C) prewetted with 50 mM Tris buffer (pH 7.50) containing 0.25% bovine serum albumin. The tubes and filter wells were rinsed with three more 4.5-ml portions of cold 0.9% NaCl. Radioactivity trapped on the filter was counted in a γ-counter (Beckman Instruments) at 70% efficiency. Each determination was performed in duplicate or triplicate. Specific binding was determined by subtracting nonspecific from total binding. In most assays, nonspecific binding was estimated by measuring filter-retained radioactivity from tubes incubated in the presence of 1 μM unlabeled angiotensin II.

Several experiments were designed to provide data to allow determination of receptor number and affinity by the LIGAND computer program (described under Data Analysis). Each of these experiments consisted of duplicate or triplicate determinations of binding in the presence of 16 different concentrations of unlabeled ligand (3×10^{-11} M to 1×10^{-5} M) and 0.3 nM ^{125}I-angiotensin II. Residual binding in the presence of 10 μM angiotensin II was subtracted from total binding to provide an initial adjustment for nonspecific binding. The program then developed final estimates of nonspecific binding.

Kinetics studies were performed by incubating 1.0 ml of tissue suspension with 1.0 ml of assay mix in polystyrene tubes (16 × 125 mm) (Falcon 2025). Aliquots (0.20 ml) were then transferred to 12 × 75 mm tubes for the filtration step. Nonspecific binding was estimated from parallel tubes containing 1 μM unlabeled angiotensin II from the beginning of incubation. To assess the rate of dissociation of radioligand from the cardiac particulate fraction, ^{125}I-angiotensin II (0.3 nM) was incubated for 60 min and then unlabeled angiotensin II was added to a final concentration of 1 μM. The decrease in specifically bound ^{125}I-angiotensin II over time (2–60 min) was assayed. To assess the rate of association of ^{125}I-angiotensin II to the receptor, the amount of radioligand specifically bound was assayed every 3–5 min for the first 30 min of incubation, then at 45 and 60 min. For associate rate experiments, the concentration of ^{125}I-angiotensin II varied from 0.2 nM to 1.2 nM.

Thin-layer chromatography. To test the purity of the radioligand and to assess degradation of ^{125}I-angiotensin II during the assay, samples of radioligand or supernatants from centrifuged assay tubes were applied to cellulose plates (Eastman 6064). The plates were devleoped by ascending thin-layer chromatography (*tert*-butyl alchol/3% NH_3, 3:1) and cut into 5-mm strips. The strips were counted in a γ-counter.

Denervation. In order to investigate the possibility that the myocardial angiotensin II receptors were located within the myocardial adrenergic nerve terminals, two rabbits were treated with a regimen of 6-hydroxydopmaine to ablate the neural terminals (32, 33). 6-Hydroxydopamine hydrobromide was dissolved in an aqueous solution of 0.1% ascorbic acid and immediately was injected i.v.: 20 mg/kg on days 1 and 2, 40 mg/kg on days 5 and 6. The rabbits were killed on day 7. Each rabbit was paired with an ascorbic acid-treated control. The

[2] The abbreviations used are: Gpp(NH)p, guanyl-5′-yl-imidodiphosphate; PMSF, phenylmethylsulfonyl fluoride; T-61, *N*-[2-(*n*-methoxyphenyl)-2-ethyl-butyl-(1)]-α-hydroxybutyramide (200 mg/ml), 4,4′-methylene-bis(cyclohexyltrimethylammonium iodide) (50 mg/ml), tetracaine HCl (5 mg/ml), and dimethyl formamide in distilled water.

norepinephrine content of each rabbit's myocardium was determined from a small apical portion as described previously (34). The binding of ^{125}I-angiotensin II (0.05–5.0 nM) to the myocardial membranes of each treated rabbit and its control was determined simultaneously.

Plasma angiotensin II levels. Plasma angiotensin II levels in five rabbits taking a standard diet with tap water *ad libitum* were measured by radioimmunoassay using standard techniques.

Adenylate cyclase activity tissue preparations. Four separate tissue preparations were used in an attempt to detect an effect of angiotensin II on myocardial adenylate cyclase activity. Each was prepared in the presence of 5 mM EDTA, as found necessary by Jard and associates (24) for inhibition of hepatic adenylate cyclase by angiotensin II. Our initial experiments were performed with the supernatant of a crude homogenate after filtration through gauze and centrifugation (1500 × *g*) for 5 minutes at 4° (Preparation A). Preparation B was identical with the binding preparation except for the inclusion of EDTA in the sucrose homogenizing medium. Preparation C was a particulate fraction prepared by the method of Woodcock and Johnston (26) except for the substitution of myocardial tissue for renal tissue. Preparation D was a sarcolemmal membrane preparation (35).

Adenylate cyclase activity assay. Adenylate cyclase activity was determined by measuring the rate of formation of [α-^{32}P]cyclic AMP from [α-^{32}P]ATP, as described by Salomon *et al.* (36). Assay mixtures (final volume 50 μliters) contained [α-^{32}P]ATP (1.5 × 10^6 dpm), 1.0 mM ATP, 25 mM Tris-HCl (pH 7.4), 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 2 mM cyclic AMP, 0.1% albumin, 10 mM theophylline, adenosine deaminase (10 units),[3] and an ATP-regenerating system consisting of 20 mM creatine phosphate and creatine phosphokinase (1 mg/ml).

In an effort to find optimal conditions for detection of an effect of angiotensin II on adenylate cyclase activity, assays were performed with two concentrations of $MgCl_2$ (0.5 and 5.0 mM) and two concentrations of GTP (10 and 100 μM). Most experiments were performed in the presence of 100 mM NaCl, since Na^+ or Li^+ appear to be necessary for the inhibition of adenylate cyclase activity in liver and kidney (24–26), but a few experiments were performed in the absence of NaCl.

In each experiment, adenylate cyclase activity was determined in triplciate under basal conditions, with angiotensin II (10^{-6} M), with isoproterenol (10^{-4} M), and with angiotensin II plus isoproterenol. Reactions were initiated by the addition of 10 μl of tissue suspension (1.35 μg–142 μg of protein, depending on the preparation). Tissue was incubated at 25° for 10 min, except for the sarcolemmal vesicles (Preparation D), which were incubated at 37° for 3 min as in the experiments of Jard *et al.* (24). The reaction was stopped by addition of 100 μl of 34 mM sodium docecyl sulfate, 40 mM ATP, and 12 mM cyclic AMP (containing 10,000 cpm [^{3}H]cyclic AMP to allow determination of recoveries of cyclic AMP after chromatographic separation). Data were expressed as picomoles of cyclic AMP formed per milligram of protein per minute.

Protein concentration assay. Protein concentrations were measured by the method of Lowry *et al.* (37), using bovine serum albumin as standards.

Data analysis. When appropriate, data were expressed as means ± standard error of the mean.

Dissociation rate constants were calculated (38) by a weighted, nonlinear least-squares program (BMDP-PAR; ref. 39). One-site and two-site models were compared, the best fit being chosen by an *F*-test. Association rate constants (38) were subjected to similar analysis. Comparison of kinetics constants under different conditions was performed using two-tailed *t*-tests. Comparison of adenylate cyclase activities in the absence and presence of angiotensin II was performed using two-tailed, paired *t*-tests. The null hypothesis was rejected when $p < 0.05$.

The equilibrium association constants and number of binding sites for the competitive binding curves were determined using the LIGAND program developed by Munson and Rodbard (40). This program uses an exact mathematical description of ligand binding to provide estimates of the equilibrium association constants (K_A) and number of receptors (N) for each ligand (labeled or unlabeled) as well as the amount of each ligand nonspecifically bound. For each curve, the program calculates standard errors of each parameter estimate. It can be used to analyze multisite systems, and provides statistically valid measurements of goodness-of-fit of the derived curves for the data. The program allows the choice of the best binding model (i.e., one site or multiple sites), and also allows testing of an experimental manipulation (e.g., addition of a cation or nucleotide) for changes in receptor affinity or number. Data from several experiments can be pooled in order to obtain the large number of points needed for estimation of binding parameters by this method. Comparisons of goodness-of-fit are made with the *F*-statistic. In this study, an *F*-statistic associated with a $p < 0.05$ was considered to indicate a statistically significant difference in the goodness-of-fit of two curves.

[3] Adenosine deaminase was not included in the initial experiments with the crude homogenate (Preparation A).

RESULTS

General Characteristics of ^{125}I-Angiotensin II Binding

Specific binding of ^{125}I-angiotensin II (0.3 nM) reached steady state in 35–45 min at 25° and remained stable through at least 90 min of incubation.

Degradation of ligand was assessed by thin-layer chromatography of supernatants from centrifuged assay tubes incubated for 60 min. Preincubation with EDTA and the inclusion of bacitracin and PMSF in the incubation mix were necessary to prevent the appearance of several peaks (presumably representing degradation products) on the developed chromatograms. In the absence of added cations or in the presence of 5 mM Mg^{2+}, there was almost no degradation of radioligand (0.3 nM ^{125}I-angiotensin II) after incubation for 60 min at 25°. No degradation was observed when 1 or 10 μM unlabeled angiotensin II and 0.3 nM ^{125}I-angiotensin II were added to the incubation medium, suggesting that no degradation occurred even when angiotensin II concentrations approached the K_m of putative degradative enzymes. Significant degradation was detected at an assay temperature of 30°, and even at 25° in the presence of 5 mM Ca^{2+} or Mn^{2+}. Therefore, all studies were performed at 25°, and Mg^{2+} was the cation used to assess divalent cation effects on the myocardial angiotensin II receptor.

Specific binding was proportional to total protein concentration over a range from 0.35 mg/ml to 6.15 mg/ml (35 μg–615 μg/assay tube). Specific binding as a fraction of the total varied according to concentrations of radioligand, cations, or nucleotides. At a concentration of 0.3 nM ^{125}I-angiotensin II and no additives, specific bionding was approximately 70% of total binding, but increased to 90–95% in the presence of 5 mM Mg^{2+}. No additive significantly altered nonspecific binding.

As shown in Fig. 1, unlabeled angiotensin II and analogues competed with ^{125}I-angiotensin II for binding to the rabbit myocardial particulate fraction with an order of potency of Sar^1-Ile^8 angiotensin II > angiotensin II > Sar^1-Ala^8 angiotensin II > angiotensin III > Ala^8 angiotensin III > angiotensin I. Interestingly, the antagonists Sar^1-Ile^8 and Sar^1-Ala^8 angiotensin II failed to compete fully with ^{125}I-angiotensin II. The competition for radioligand binding sites by angiotensin II and Sar^1-Ile^8 angiotensin II was examined more closely in another series of experiments (described below).

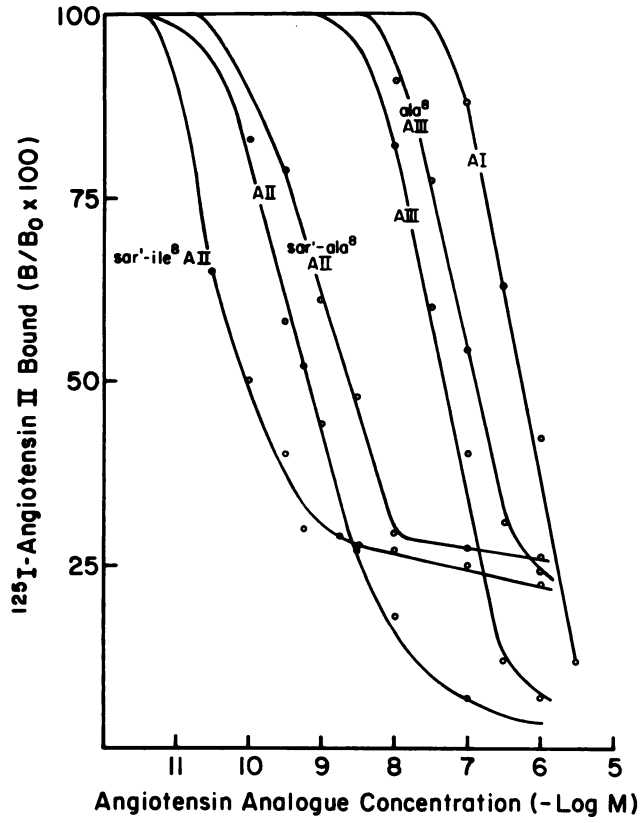

FIG. 1. *Competition for ^{125}I-angiotensin II binding sites in rabbit myocardial particulate fraction by angiotensin II and its analogues*
^{125}I-Angiotensin II (0.3 nM) was incubated for 60 min at 25° with 250–350 μg of protein. *B* and B_0 represent the binding in the presence and absence, respectively, of unlabeled peptide. Results are means of three determinations. *AI, AII,* and *AIII* represent angiotensin I, II, and III, respectively.

In the two rabbits treated with 6-hydroxydopamine hydrobromide to ablate intramyocardial adrenergic nerve terminals, myocardial norepinehrine levels were less than 10% of levels in control rabbits. The binding of ^{125}I-angiotensin II (0.05–6.0 nM) as determined by Scatchard analysis was the same as that in the controls, suggesting that adrenergic terminals did not contribute significantly to the total population of binding sites.

Determination of Binding Parameters

Competition for ^{125}I-angiotensin II binding sites by unlabelled angiotensin II or Sar^1-Ile^8 angiotensin II was analyzed with the LIGAND program. A composite of the computer-generated angiotensin II competition curves under the various conditions investigated is displayed in Fig. 2. Analysis of the three competition curves obtained in the absence of added cations or nucleotides showed in each case that the binding data were better fitted ($p < 0.001$) to a two-site model than to a single-site model. The data indicate the presence of a high-affinity, low-capacity site and a low-affinity, high-capacity site. To obtain the optimal estimate of the binding parameters, the data from the three experiments were combined as described by Munson and Rodbard (40). The estimates obtained were as follows: $K_{A1} = 2.4 \pm 0.6 \times 10^9$ M^{-1} ($K_{D1} = 0.42$ nM), $N_1 = 7.8 \pm 0.8$ fmoles/mg of protein; $K_{A2} = 9.6 \pm 0.6 \times 10^6$ M^{-1} ($K_{D2} = 104$ nM), $N_2 = 219 \pm 128$ fmoles/mg of protein (Table 1).

Analysis of two competition curves (96 data points) of ^{125}I-angiotensin II by Sar^1-Ile^8 angiotensin II (10^{-12} M–10^{-5} M) indicated that the antagonist bound to two sites, one of low capacity (approximately 10 fmoles/mg of protein) with very high affinity ($K_{A1} = 8 \times 10^9$ M^{-1}). The second site displayed an extremely low affinity for Sar^1-Ile^8 angiotensin II ($K_{A2} = 10^5$ M^{-1}). Probably because of the very low affinity for antagonist displayed by this site, the parameter estimates were considered in the program to be "ill-conditioned," and standard errors were not obtainable.

Effect of Cations and Nucleotides on Angiotensin II Receptor Binding Parameters

Monovalent cations. All of the monovalent cations tested at concentrations from 10 to 150 mM caused mod-

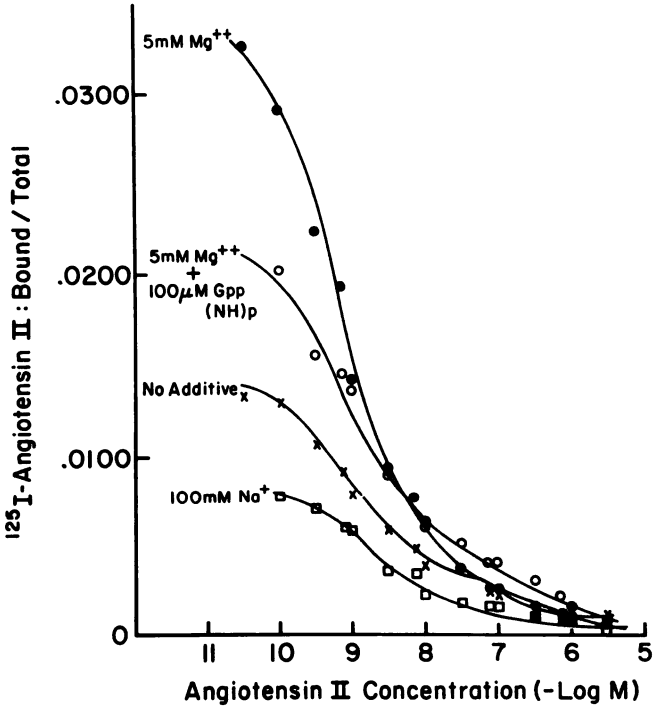

FIG. 2. *Effects of Na^+, Mg^{2+}, and Gpp(NH)p on competition curves of ^{125}I-angiotensin II bound to rabbit cardiac membranes*
Unlabeled angiotensin II was added at concentrations between 0.03 nM and 3 μM to assay tubes containing 0.3 nM ^{125}I-angiotensin II and 250–350 μg of protein under the four sets of conditions shown. Nonspecific binding for these experiments was initially estimated as filter-retained radioactivity in the presence of 10 μM unlabeled angiotensin II, but further adjustments for nonspecific binding were made by the LIGAND program (40). Ten experiments were performed. For each condition, data points from all relevant experiments were combined in order to obtain the large number of points required for estimation of binding parameters. The curves represent the best nonlinear regression fits of the data points. Each point represents the mean of four to seven determinations for bound/total at a particular concentration of unlabeled angiotensin II. Thus, between 72 and 114 determinations of binding contributed to each of the computer-generated curves. For all but five plotted points, the maximal difference between individual values of bound/total and the mean value was less than 0.0015. The estimates of binding parameters describing these pooled curves are shown in Table 1.

TABLE 1

Equilibrium binding parameters for rabbit myocardial membrane angiotensin II receptors

Under the four sets of conditions shown, equilibrium association constants (K_A) and receptor density (N) were determined using the LIGAND program, which developed the curves shown in Fig. 2 (as described under Data Analysis). Data are expressed as parameter estimate ± standard error of the estimate. Brackets connect pairs of parameter estimates compared statistically; results of statistical comparisons are given in parentheses adjacent to the corresponding brackets. Neither K_{A2} nor N_2 varied significantly with the four conditions. NS, Not significant ($p > 0.05$).

Additive(s)	K_{A1}	N_1	K_{A2}	N_2
	M^{-1}	*fmoles/mg protein*	M^{-1}	fmoles/mg protein
None	$2.4 \pm 0.6 \times 10^9$	7.8 ± 0.8	$9.6 \pm 0.6 \times 10^6$	219 ± 128
	(NS)	($p < 0.05$)		
Na^+, 100 mM	$1.5 \pm 0.5 \times 10^9$	2.6 ± 0.7	$5.0 \pm 3.1 \times 10^6$	199 ± 13
	(NS)	($p < 0.05$)		
Mg^{2+}, 5 mM	$1.8 \pm 0.3 \times 10^9$	14.4 ± 1.5	$11 \pm 13 \times 10^6$	143 ± 152
	($p < 0.05$)	(NS)		
Mg^{2+}, 5 mM, + Gpp(NH)p, 100 μM	$1.0 \pm 0.2 \times 10^9$	15.1 ± 2.2	$1.0 \pm 2.2 \times 10^6$	1180 ± 2610

est decreases in specific binding of ^{125}I-angiotensin II. At a concentration of 150 mM, Na^+, K^+, Li^+, and NH_4^+ reduced binding of 0.3 nM ^{125}I-angiotensin II to 69 ± 3%, 74 ± 2%, 60 ± 6%, and 86 ± 4% of control, respectively.[4] The effects of Na^+ were examined in more detail. Analysis of the competition for ^{125}I-angiotensin II binding sites by unlabeled angiotensin II in the presence of 100 mM NaCl (three pooled experiments) demonstrated no significant change in K_{A1} or K_{A2}, no change in the number of low-affinity sites, but a 67% reduction ($p < 0.05$) in the number of high-affinity sites (to 2.6 ± 0.7 fmoles/mg of protein) (Table 1; Fig. 2).

Divalent cations. Divalent cations stimulated binding of ^{125}I-angiotensin II to the rabbit myocardial particulate fraction with an order of potency of $Mg^{2+} > Mn^{2+} > Ca^{2+}$. However, both Mn^{2+} and Ca^{2+} induced degradation of radioligand. Magnesium caused an increase in binding to a plateau approximately twice control binding at concentrations between 1 and 20 mM. Analysis of three pooled competition curves (Fig. 2) in the presence of 5 mM Mg^{2+} showed that the cation caused no significant change in K_{A1} or K_{A2}, no change in the number of low-affinity sites, but a doubling ($p < 0.05$) of the number of high-affinity sites (to 14.4 ± 1.5 fmoles/mg of protein) (Table 1).

Nucleotides. In the absence of added Mg^{2+}, nucleotides failed to exert a consistent effect on ^{125}I-angiotensin II binding. The increase in specific binding induced by 5 mM Mg^{2+} was attenuated in a concentration-related manner by nucleotides with an order of potency of Gpp(NH)p > GTP > GDP > ATP > GMP. In the presence of 5 mM Mn^{2+}, 100 μM Gpp(NH)p caused a reduction in the specific binding of 0.3 nM ^{125}I-angiotensin II to 60% of specific binding in the presence of 5 mM Mg^{2+} alone. Two competition curves performed in the presence of 5 mM Mg^{2+} and 100 μM Gpp(NH)p were analyzed in detail (Fig. 2). As shown in Table 1, there was a significant decrease ($p < 0.05$) in K_{A1} (to $1.0 \pm 0.2 \times 10^9$ M^{-1}). Compared with K_{A2} in the presence of 5 mM Mg^{2+}, Gpp(NH)p induced an 11-fold decrease in K_{A2}, but the estimates of K_{A2} were sufficiently imprecise that even this apparently large change was not statistically significant. The number of high-affinity sites was unchanged (15.1 ± 2.2 fmoles/mg of protein). The number of low-affinity sites appeared to increase (1180 fmoles/mg of protein), but the standard error was too great (2610 fmoles/mg of protein) to allow any conclusion to be drawn concerning the number of low-affinity sites.

[4] Control binding: specific binding in the absence of added cations or nucleotides.

Effect of Mg^{2+} and Gpp(NH)p on Kinetic Parameters of ^{125}I-Angiotensin II binding

Dissociation rates. The analysis of the kinetic data is summarized in Table 2 and Fig. 3. Four dissociation curves performed in the absence of cations or nucleotides were fitted to exponential decay curves. In each case, the best fit was obtained with a two-component model representing a quickly dissociating ($k_{-1} = 0.19 \pm 0.02$ min^{-1}) and a slowly dissociating ($k_{-1} = 2.5 \pm 2.1 \times 10^{-3}$ min^{-1}) site. Under these conditions, 58 ± 3% of the sites were slowly dissociating.

In the presence of 5 mM Mg^{2+} (three experiments), there was little change in the dissociation rate of either the rapidly dissociating component ($k_{-1} = 0.12 \pm 0.02$ min^{-1}), or the slowly dissociating component ($k_{-1} = 3.4 \pm 0.5 \times 10^{-3}$ min^{-1}). However, the fraction of slowly dissociating receptors rose to 76 ± 3% ($p < 0.01$).

In the presence of 5 mM Mg^{2+} and 100 μM Gpp(NH)p (three experiments), the dissociation rates of the rapid and slow components increased ($p < 0.01$) to 0.41 ± 0.09 min^{-1} and $10.4 \pm 1.7 \times 10^{-3}$ min^{-1}, respectively. The fraction of slowly dissociating receptors (74 ± 3%) was the same as that in the presence of 5 mM Mg^{2+} alone.

The dissociation rate data are consistent with the equilibrium data if it is assumed that the high- and low-affinity sites correspond to the slowly and rapidly dissociating components. Magnesium induced little or no change in dissociation rates, but binding to the high-affinity site constituted a greater fraction of total binding, as expected from the equilibrium data which indicated an increase in high affinity receptor number. Similarly, the addition of Gpp(NH)p to Mg^{2+} caused significant acceleration of both dissociation rates, consistent with the decrease in equilibrium association constants.

TABLE 2

Effect of Mg^{2+} or Mg^{+} with Gpp(NH)p on dissociation rate constants of the rabbit myocardial membrane angiotensin II receptor

Details of experiments are given under Methods and in the Legend to Fig. 3. Data are expressed as parameter estimate ± standard error of the estimate. Brackets connect pairs of parameter estimates compared statistically; results of statistical comparisons are given in parentheses adjacent to the corresponding brackets. NS, Not significant ($p > 0.05$)

Additive(s)	k_{-1} (fast)	k_{-1} (slow)	% (slow/total)[a]
	min^{-1}	min^{-1}	
None	0.19 ± 0.02 (NS)	$2.5 \pm 2.1 \times 10^{-3}$ (NS)	58 ± 3 ($p < 0.05$)
Mg^{2+}, 5 mM	0.12 ± 0.02 ($p < 0.05$)	$3.4 \pm 0.5 \times 10^{-3}$ ($p < 0.05$)	76 ± 3 (NS)
Mg^{2+}, 5 mM + Gpp(NH)p, 100 μM	0.41 ± 0.09	$10.4 \pm 1.7 \times 10^{-3}$	74 ± 3

[a] Percentage (slow/total) represents the percentage of total bound ligand which is bound to the slowly dissociating (presumably high-affinity) site in the presence of 0.3 nM ^{125}I-angiotensin II.

Association rates. In order to make an independent determination of K_A based on analysis of association and dissociation rates ($K_A = k_1/k_{-1}$), we attempted to fit association curves to an appropriate two-component exponential equation, but were unsuccessful. However, if we assumed that the association rates of the two components were equal, k_1 usually was assigned a value in the range of 1×10^6 to 1×10^7 M^{-1} sec^{-1}. Further analysis suggested that the association rates were, in fact, similar. Assuming that the slowly dissociating component corresponds to the high-affinity site demonstrated by equilibrium analysis and that the rapid component corresponds to the low-affinity site, we estimated association rate constants: $k_1 = K_A \times k_{-1}$. In the absence of additives, the association constants for high- and low-affinity sites were 5.9×10^6 M^{-1} sec^{-1} and 1.8×10^6 M^{-1} sec^{-1}, respectively.

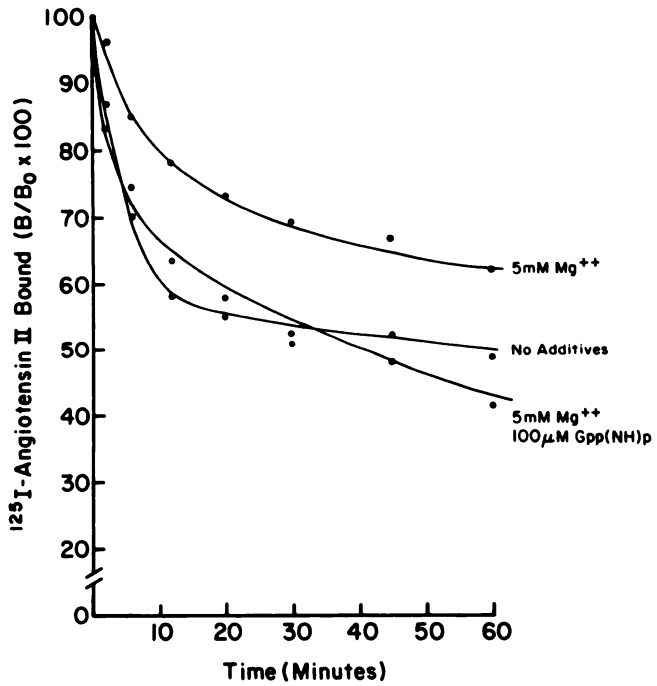

FIG. 3. *Effects of Mg^{2+} and Gpp(NH)p on dissociation of ^{125}I-angiotensin II (0.3 nM) from rabbit myocardial membranes*

Tissue was incubated in the absence of additives, in the presence of 5 mM Mg^{2+}, or in the presence of 5 mM Mg^{2+} with 100 μM Gpp(NH)p. After incubation at 25° for 60 min, unlabeled angiotensin II was added (final concentration 1 μM) and the decline in specific binding of ^{125}I-angiotensin II was followed over time. B_0 is specific binding just before addition of unlabeled angiotensin II; B is specific binding at the times indicated. Each experimental determination was performed in duplicate, and each experiment was performed three or four times. Plotted points represent observed mean values of B/B_0; lines represent computer-derived curves providing the best fit of the data by weighted nonlinear regression (as described under Data Analysis). In each case, the data are best described by a two-component dissociation model. Dissociation rate constants are displayed in Table 2.

Angiotensin II and Adenylate Cyclase

Since guanine nucleotides regulate both agonist binding and coupling to adenylate cyclase for a number of receptors (41), we investigated the possibility that the guanine nucleotide effects on the binding of angiotensin II to its myocardial receptor might reflect events involved in the coupling of this receptor to adenylate cyclase. The results were qualitatively the same in the four tissue preparations, regardless of the presence or absence of NaCl, the concentrations of $MgCl_2$ (0.5 or 5.0 mM), or the concentration of GTP (10 or 100 μM): angiotensin II (10^{-6} M) had no influence on basal or isoproterenol-stimulated adenylate cyclase activity. In a total of 19 experiments

TABLE 3

Lack of effect of angiotensin II on basal or isoproterenol-stimulated adenylate cyclase activity

These experiments were performed with 100 mM NaCl, 0.5 mM $MgCl_2$, and 100 μM GTP, as described under Methods. Preparation A (crude homogenate) was not investigated under this set of conditions. Several other experiments with different concentrations of NaCl, $MgCl_2$, or GTP also failed to show an effect of angiotensin II on adenylate cyclase activity.

Tissue preparation[a]	Adenylate cyclase activity			
	Basal	Angiotensin II (10^{-6} M)	Isoproterenol (10^{-4} M)	Angiotensin II + isoproterenol
	pmoles/mg protein/min			
A	—	—	—	—
B (2)	58.3	68.4	97.7	93.9
C (1)	17.5	17.5	22.7	23.9
D (2)	274	288	363	364

[a] A, Preparation A (crude homogenate); B, binding preparation; C, particulate fraction (26); D, sarcolemma (35). Numbers in parentheses indicate number of experiments.

(with various combinations of NaCl, $MgCl_2$, and GTP concentrations), the average ratio of adenylate cyclase activity in the presence of angiotensin II to basal activity was 1.03, with a range from 0.9 to 1.2. In the presence of isoproterenol (10^{-4} M), the average ratio of adenylate cyclase activity in the presence versus the absence of angiotensin II was 0.99, with a range from 0.9 to 1.1 (17 experiments). Results for one set of conditions (100 mM NaCl, 0.5 mM $MgCl_2$, 100 μM GTP) are given in Table 3.

Plasma Angiotensin II Measurements

Plasma angiotensin II concentrations in five rabbits were between 0.04 and 0.14 nM.

DISCUSSION

The ligand ^{125}I-angiotensin II identifies binding sites in rabbit ventricular myocardium with the specificity expected of physiological angiotensin II receptors. These receptors, which differ in several respects from those which we have described previously in rat mesenteric arteries (17), have a number of salient features: (a) there are two classes of receptors, one of high affinity, low capacity and the other of low affinity, high capacity; (b) Na^+ decreases and Mg^{2+} increases the number of high-affinity sites without changing the affinity of these sites; (c) the GTP analogue, Gpp(NH)p, decreases the affinity of both sites without changing receptor number, suggesting that the receptor is associated with a guanine nucleotide regulatory protein; and (d) the guinine nucleotide regulatory protein does not link the myocardial angiotensin II receptor to adenylate cyclase.

The ventricular particulate fraction may have contained vascular or neural elements in addition to myocyte membranes. Treatment with 6-hydroxydopamine reduced myocardial norepinephrine content by 90% and probably caused ablation of intramyocardial adrenergic terminals (32, 33), which may possess angiotensin II receptors (8). Nevertheless, binding of ^{125}I-angiotensin II was not reduced, suggesting that neural terminals did not constitute a significant portion of the particulate fraction receptor population. We cannot entirely eliminate the possibility that neural membrane angiotensin II receptors persisted despite pharmacological disruption of the nerve terminals themselves, but consider this unlikely. To evaluate the possibility that the receptors were vascular, we prepared a particulate fraction of rabbit mesenteric arteries (using techniques similar to those we have used to prepare rat mesenteric artery tissue) (17). After the rabbit mesenteric artery membranes were exposed to the same EDTA/PMSF preincubation and the same assay mix as utilized in the heart preparation, binding of ^{125}I-angiotensin II was unaffected by Na^+,[5] whereas in heart membranes Na^+ attenuated radioligand binding. These results in the rabbit mesenteric arteries are in agreement with previous results in rabbit aorta in which Na^+ did not affect [^{3}H]-angiotensin II binding (13). In comparing cardiac and mesenteric arterial preparations from the rat, we found that Na^+ inhibited radioligand binding to heart membranes[5] but stimulated binding in vascular tissue (17). The different effects of Na^+ on binding of ^{125}I-angiotensin II to cardiac and arterial preparations in two species suggest that the receptor assayed in the myocardial particulate fraction was not primarily vascular in origin.

The order of potency of angiotensin analogues in competing for ^{125}I-angiotensin II binding sites (Fig. 1) generally parallels the potency order of the analogues as positive inotropic agents (3, 4). Although some physiological studies have shown angiotensin II and angiotensin III to be equipotent, the bulk of the evidence indicates that angiotensin II is approximately 5-fold more potent in heart than angiotensin III (11). The higher affinity for angiotensin II than for angiotensin III in rabbit myocardial membranes observed in this study corresponds reasonably well with those pharmacological observations.

The two classes of binding sites differ strikingly in their affinities (K_{D1} = 0.42 nM and K_{D2} = 104 nM). The affinity of the high-affinity site is sufficient to allow occupancy of approximately 20% of those sites at the circulating concentrations of angiotensin II (0.04 nM–0.14 nM) which we measured in salt- and volume-replete rabbits. Studies in cat papillary muscles (1, 2, 4) as well as perfused rabbit hearts (3) have shown the onset of noticeable inotropic effects at angiotensin II concentrations of 0.1 nM–1.0 nM and 50% maximal effect at 5 nM–10 nM. Increases in myocardial cell electrical coupling, which have been invoked to explain the inotropic effects (7), occur at similar concentrations of angiotensin II. These effects on contractility (5) and electrical coupling (7) are inhibited by Sar^1-Ala^8 angiotensin II, which (like Sar^1-Ile^8 angiotensin II) avidly competes with angiotensin II for the high-affinity site but not for the low-affinity site in rabbit myocardium. It is likely, therefore, that the high-affinity receptor mediates the inotropic effects described by others. The low-affinity, high-capacity binding site is distinguished by the inability of the Sar^{-1}-Ala^8 and Sar^1-Ile^8 analogues of angiotensin II to interact with this site. The nature and physiological function of this low-affinity site are unknown. It seems unlikely that the low-affinity binding site is located on degradative enzymes, since no angiotensin II breakdown was noted even in the presence of 10 μM angiotensin II. Although we cannot exclude the possibility that the two sites represent different affinity states of the same receptor, the striking differences in specificity with respect to the sarcosine analogues seem to make this unlikely. The two sites may exist at different locations in the myocardium. The number of low-affinity binding sites is sufficiently great that at physiologically attainable angiotensin II concentrations, 20–40% of the total bound peptide may be bound to these sites. The regulation by Gpp(NH)p of the affinity of low-affinity site, suggested by equilibrium data and corroborated by the kinetic data, supports the notion that this site may subserve a presently undefined physiological function.

The decrease of binding of ^{125}I-angiotensin II to its myocardial receptor due to Na^+ is not specific for this monovalent cation, since Li^+, K^+, and NH_4^+ also inhibit binding. In other tisues, Na^+ either exerts no effect on angiotensin II binding, as in rabbit vascular smooth muscle (13), rabbit (16) and rat (19) uterus, or it stimulates binding, as in bovine brain (16) and adrenal cortex (11,

[5] Unpublished observations.

16) and in rat adrenal cortex (19), liver (18), and mesenteric artery (17). In liver, the increase in binding is due to an increase in the number of high-affinity sites (18), whereas in mesenteric artery (17) and adrenal cortex (19) it is due to an increase in receptor affinity. The mechanism whereby Na^+ reduces the number of high-affinity sites in rabbit myocardium is not clear, but this response by these receptors provides yet another example of the heterogeneity of angiotensin II receptors in different tissues and species. On the other hand, these data provide further evidence that a general feature of angiotensin II receptors is their association with cation-sensitive sites (11).

The enhancement of ^{125}I-angiotensin II binding to the myocardial angiotensin II receptor by Mg^{2+} is similar to the effects of divalent cations on hormone binding to a number of receptors (ref. 41; discussed in ref. 17). Magnesium increases the affinity of the rat mesenteric artery angiotensin II receptor (17). By contrast, both the rabbit myocardial angiotensin II receptor and the rat hepatic angiotensin II receptor (18) increase the number of high-affinity receptors in response to Mg^{2+}. The significance of these differences in the manner in which divalent cations regulate angiotensin II receptors is not clear. It is likely, however, that divalent cations are important in the events associated with angiotensin II receptor function.

In the presence of Mg^{2+}, guanine nucleotides decrease the affinity of both classes of angiotensin II binding sites in rabbit myocardium. Although the estimates of the binding parameters for the high-affinity site can be determined with good precision, the estimates for the low-affinity site in both the absence and presence of Gpp(NH)p are imprecise. The decrease in affinity of both sites suggested by the equilibrium data is supported by the significant acceleration of both components of dissociation in the presence of Gpp(NH)p. These data contrast with those from the rat liver, in which guanine nucleotides do not alter the affinity of the high-affinity angiotensin II binding sites, but dramatically reduce their number (25). By either mechanism, however, guanine nucleotides reduce the amount of agonist bound to the angiotensin II receptor at a given concentration.

The effects of Na^+, Mg^{2+}, and Gpp(NH)p on binding to the myocardial angiotensin II receptor provide interesting evidence that this receptor may be coupled to adenylate cyclase. Sodium and other monovalent cations inhibit, and Mg^{2+} stimulates, agonist binding to several receptors (e.g., *alpha*$_2$-adrenergic, opiate, adenosine) which mediate inhibition of adenylate cyclase activity (ref. 41: discussed in ref. 17). Furthermore, the effects of Gpp(NH)p on myocardial angiotensin II receptor affinity and dissociation rate are strikingly similar to the effects of guanine nucleotides in systems linked to adenylate cyclase activation or inhibition (41). Angiotensin II inhibits adenylate cyclase activity in liver membranes (24, 25) and renal cortical homogenates (26) treated with EDTA and incubated with Na^+ or Li^+. Similarly, angiotensin II inhibits adenylate cyclase activity in intact hepatocytes (25). Nevertheless, we were unable to detect effects of angiotensin II on basal or isoproterenol-stimulated adenylate cyclase activity in several tissue preparations treated with EDTA and incubated with Na^+, thus confirming the early report of Goodfriend *et al.* (22). In this respect, the myocardial angiotensin II receptor is similar to the adrenal cortical and vascular smooth muscle angiotensin II receptors, which are regulated by guanine nucleotides (12, 17) but probably not linked to adenylate cyclase (23, 27). The regulation by guanine nucleotides of the affinity of angiotensin II receptors which are not coupled to adenylate cyclase raises the possibility that these receptors are associated with guanine nucleotide regulatory proteins different from those involved in receptor coupling to adenylate cyclase (41).

Our data differ from those of Mukherjee *et al.* (29), who characterized the angiotensin II receptor in a bovine sarcolemmal preparation. Those authors found only a single high-affinity site (K_D = 2.2 nM), but Sar^1-Ala^8 angiotensin II and Sar^1-Ile^8 angiotensin II competed for ^{125}I-angiotensin II binding sites with respective potencies of only 10% and 2% of that of angiotensin II. It is possible that these differences reflect differences in species or membrane preparations. It is also possible that technical differences in approach account for the disparate findings. For example, when we initially examined receptor number and affinity in rabbit myocardium, we assayed ^{125}I-angiotensin II binding at concentrations between 0.05 and 5.0 nM and analyzed the data by Scatchard analysis, an approach similar to that utilized by Mukherjee *et al.* (29). By that method, we identified (and reported in preliminary form) a single class of binding sites with K_D = 1.4 nM (30). This finding became increasingly difficult to reconcile with the two-component dissociation kinetics. It was only by using the LIGAND program to analyze ^{125}I-angiotensin II displacement by angiotensin II concentrations as high as 10^{-5} M that we were able to identify the second site. Even if the second site is biologically inert, it must be accounted for in investigations of the effects of biochemical interventions on binding of ligands to the myocardial angiotensin II receptor.

In summary, our investigation has utilized reiterative computer analysis techniques to characterize the binding of ^{125}I-angiotensin II to its receptor in a rabbit myocardial particulate fraction. Both equilibrium and kinetic data indicate the presence of two sites. The first site possesses the high affinity and specificity expected of a physiological angiotensin II receptor. The second site binds angiotensin II with low affinity. Monovalent and divalent cations modulate the number of high-affinity sites, and guanine nucleotides reduce the affinities of both sites, which are not linked to adenylate cyclase. Delineation of the role of receptor-associated cation and guanine nucleotide binding sites in receptor activation requires further investigation.

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REFERENCES

1. Koch-Weser, J. Myocardial actions of angiotensin. *Circ. Res.* **14**:337–344 (1964).
2. Dempsey, P. J., Z. T. McCallum, K. M. Kent, and T. Cooper. Direct myocardial effects of angiotensin II. *Am. J. Physiol.* **220**:477–481 (1971).
3. Bonnardeux, J. L., and D. Regoli. Action of angiotensin and analogues on the heart. *Can. J. Physiol. Pharmacol.* **52**:50–60 (1974).
4. Trachte, G. J., and A. M. Lefer. Inotropic and vasoactive effects of the naturally occuring angiotensins in isolated cat cardiac muscle and coronary arteries. *Res. Commun. Chem. Pathol. Pharmacol.* **25**:419–427 (1979).
5. Freer, R. J., A. J. Pappano, M. J. Peach, K. T. Bing, M. J. McClean, S. Vogel, and N. Sperelakis. Mechanism for the positive inotropic effect of angiotensin II on isolated cardiac muscle. *Circ. Res.* **39**:178–183 (1976).
6. Kass, R. S., and M. L. Blair. Effects of angiotensin II on membrane current in cardiac Purkinje fibers. *J. Mol. Cell. Cardiol.* **13**:797–809 (1981).
7. Hermsmeyer, K. Angiotensin II increases electrical coupling in mammalian ventricular myocardium. *Circ. Res.* **74**:524–529 (1980).
8. Blumberg, A. L., J. A. Ackerly, and M. J. Peach. Differentiation of neurogenic and myocardial angiotensin II receptors in isolated rabbit atria. *Circ. Res.* **36**:719–726 (1975).
9. Regoli, D., W. K. Park, and F. Rioux. Pharmacology of angiotensin. *Pharmacol. Rev.* **26**:69–123 (1974).
10. Peach, M. J. Renin-angiotensin system: biochemistry and mechanisms of action. *Physiol. Rev.* **57**:313–370 (1977).
11. Glossmann, H., A. Baukal, and K. J. Catt. Cation dependence of high-affinity angiotensin II binding to adrenal cortex receptors. *Science (Wash. D.C.)* **185**:281–283 (1974).
12. Glossmann, H., A. Baukal, and K. J. Catt. Angiotensin II receptors in bovine adrenal cortex: modification of angiotensin II binding by guanyl nucleotides. *J. Biol. Chem.* **249**:664–666 (1974).
13. Devynck, M.-A., and P. Meyer. Angiotensin receptors in vascular tissue. *Am. J. Med.* **61**:758–767 (1976).
14. Blanc, E., J. Sraer, J.-D. Sraer, L. Baud, and R. Ardaillou. Ca^{2+} and Mg^{2+} dependence of angiotensin II binding to isolated rat renal glomeruli. *Biochem. Pharmacol.* **27**:517–524 (1978).
15. Bennett, J. P., Jr., and S. H. Snyder. Angiotensin II binding to mammalian brain membranes. *J. Biol. Chem.* **251**:7423–7430 (1976).
16. Bennett, J. P., Jr., 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).
17. Wright, G. B., R. W. Alexander, L. S. Ekstein, and M. A. Gimbrone, Jr. Sodium, divalent cations, and guanine nucleotides regulate the affinity of the rat mesenteric artery angiotensin II receptor. *Circ. Res.* **50**:462–469 (1982).
18. Campanile, C. P., J. K. Crane, M. J. Peach, and J. C. Garrison. The hepatic angiotensin II receptor. I. Characterization of the membrane-binding site and correlation with physiological response in hepatocytes. *J. Biol. Chem.* **257**:4951–4958 (1982).
19. 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* **4(Supp III)**:III-79–III-84 (1982).
20. Moore, G. J. Multiple forms of angiotensin receptors in uterine smooth muscle. *Can. J. Physiol. Pharmacol.* **58**:1373–1376 (1980).
21. Caldicott, W. J. H., and N. K. Hollenberg. Offset of actions of angiotensin II, angiotensin III, and their analogue antagonists in renal and femoral vascular beds: further evidence for different vascular angiotensin receptors. *Life Sci.* **24**:503–512 (1979).
22. Goodfriend, T. L., F. Fyhrquist, F. Gutmann, E. Knych, H. Hollemans, D. Allmann, K. Kent, and T. Cooper. Clinical and conceptual uses of angiotensin receptors, in *Hypertension '72* (J. Genest and E. Koiw, eds.). Springer-Verlag, New York, 549–563 (1972).
23. Fujita, K., G. Aguilera, and K. J. Catt. The role of cyclic AMP in aldosterone production by isolated zona glomerulosa cells. *J. Biol. Chem.* **254**:8567–8574 (1979).
24. Jard, S., B. Cantau, and K. H. Jacobs. Angiotensin II and α-adrenergic agonists inhibit rat liver adenylate cyclase. *J. Biol. Chem.* **256**:2603–2606 (1981).
25. Crane, J. K., C. P. Campanile, and J. C. Garrison. The hepatic angiotensin II receptor II: effect of guanine nucleotides and interaction with cyclic AMP production. *J. Biol. Chem.* **257**:4959–4965 (1982).
26. Woodcock, E. A., and C. I. Johnston. Inhibition of adenylate cyclase by angiotensin II in rat renal cortex. *Endocrinology* **111**:1687–1691 (1982).
27. Penit, J., M. Faure, and S. Jard. Vasopressin and angiotensin II receptors in rat aortic smooth muscle cells in culture. *Am. J. Physiol.* **244**:E72–E82 (1982).
28. Lin, S.-Y., and T. L. Goodfriend. Angiotensin receptors. *Am. J. Physiol.* **218**:1319–1328 (1970).
29. Mukherjee, A., P. V. Kulkarni, Z. Haghani, and J. L. Sutko. Identification and characterization of angiotensin II receptors in cardiac sarcolemma. *Biochem. Biophys. Res. Commun.* **105**:575–581 (1982).
30. Wright, G. B., R. W. Alexander, L. S. Ekstein, and M. A. Gimbrone, Jr. Characterization of the rabbit myocardial angiotensin II receptor. *Circulation* **64(Supp IV)**:IV-285 (1981).
31. Baker, K., G. Trachte, C. Campanile, J. Young, and M. Peach. Characterization of the rabbit angiotensin II myocardial receptor. *Fed. Proc.* **41**:1708 (1982).
32. Maling, H. M., J. H. Fleisch, and W. F. Saul. Species differences in aortic responses to vasoactive amines: the effects of compound 48/80, cocaine, reserpine, and 6-hydroxydopamine. *J. Pharmacol. Exp. Ther.* **176**:672–683 (1971).
33. Kostrzewa, R. M., and D. M. Jacobowitz. Pharmacological actions of 6-hydroxydopamine. *Pharmacol. Rev.* **26**:199–288 (1974).
34. Ciarlone, A. E. Further modification of a fluorometric method for analyzing brain amines. *Microchem. J.* **23**:9–12 (1978).
35. Jones, L. R., H. R. Besch, Jr., J. W. Fleming, M. M. McConnaughey, and A. M. Watanabe. Separation of vesicles of cardiac sarcolemma from vesicles of cardiac sarcoplasmic reticulum. *J. Biol. Chem.* **254**:530–539 (1979).
36. Salomon, Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548 (1974).
37. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
38. Williams, L. T., and R. J. Lefkowitz. Theory of ligand-receptor interactions, in *Receptor Binding Studies in Adrenergic Pharmacology*. Raven Press, New York, 27–41 (1978).
39. Ralston, M. PAR: derivative-free nonlinear regression, in *BMDP Statistical Software*. University of California Press, Berkeley, 305–327 (1981).
40. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**:220–239 (1980).
41. Rodbell, M. The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature (Lond.)* **284**:17–22 (1980).

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