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Evidence for Agonist-Induced Interaction of Angiotensin Receptor with a Guanine Nucleotide-Binding Protein in Bovine Adrenal Zona Glomerulosa

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SUMMARY

Previous studies have documented that high affinity binding of [^{125}I]angiotensin II to adrenal cortex receptors was modulated by guanine nucleotides. Since in other receptor systems, similar properties of hormone-receptor interactions were shown to be specific for agonists, we studied the differential binding characteristics of agonists and antagonists to this receptor using a new radiolabeled antagonist [^{125}I] [Sar^1,Ile^8] angiotensin II. Receptor saturation studies indicate that the antagonist is binding to a homogeneous population of sites with a K_d of 0.6–2.0 nM and with a receptor density around 1 pmol/mg of protein. Competition curves using unlabeled antagonists are characterized by a slope factor of 1 and a single K_d of 1–3 nM. Addition of guanylylimidodiphosphate to the assay is absolutely without effect on radiolabeled antagonist binding. In contrast, competition curves using the full agonists angiotensin II, [Sar^1]angiotensin II, angiotensin III, and [des-Arg]angiotensin III display slope factors of 0.79, 0.87, 0.70, and 0.84, respectively. These curves can be explained by two apparent forms of the receptor having high and low affinity for the agonist. The higher affinity form associated with these four agonists is characterized by a K_d of 1.2 nM, 0.25 nM, 0.8 nM, and 3 μM, and corresponds to 60, 56, 42, and 25% of angiotensin II-binding sites, respectively. The other form displays 13- to 33-fold lower affinity. Addition of guanine nucleotide to the assay results in a 2–4-fold shift to the right and a steepening (slope factor 0.9–1.0) of agonist competition curves. Angiotensin II receptors, occupied by the full agonist [^{131}I] [Sar^1] angiotensin II or by the antagonist [^{125}I] [Sar^1, Ile^8]angiotensin II, were then solubilized with the nonionic detergent octylglucoside. Dissociation of the agonist [^{131}I] [Sar^1] angiotensin II from solubilized receptors is enhanced by guanylylimidodiphosphate or sodium acetate, while dissociation of the antagonist [^{125}I] [Sar^1, Ile^8]angiotensin II displays little sensitivity towards guanine nucleotides or increased ionic strength. Inclusion of bile salts in the solubilization medium preferentially destabilizes receptor-bound agonist, presumably by interfering with protein-protein interactions required for high affinity agonist binding. Separation of radiolabeled agonist and antagonist-occupied solubilized receptor complexes by steric exclusion high performance liquid chromatography reveals that the agonist-occupied receptor complex behaves as a larger protein than the antagonist-occupied receptor complex. These results indicate that the receptor-binding properties formerly documented for angiotensin II are specific for agonists and that the agonist-specific high affinity receptor complex appears to include a guanine nucleotide-binding protein involved in regulation of receptor affinity and possibly of its function.

INTRODUCTION

The mechanism of action of angiotensin II on the adrenal cortex is not yet fully understood, but it appears to involve several molecular events in the plasma membrane of zona glomerulosa cells (1). Calcium has been shown to be necessary for angiotensin II stimulation of aldosterone production (2). The hormone also appears to stimulate phosphatidylinositol turnover in the adrenal cortex (3). A role for cyclic AMP has, however, not been established. Fujita *et al.* (4) have shown that, in intact subcapsular cells, angiotensin II promoted aldosterone production without any stimulatory effect on cyclic AMP intracellular level. In fact, angiotensin II appears to inhibit adenylate cyclase activity in adrenal cortex (5).

Pioneering studies of Glossmann and co-workers (6,

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7) on the direct binding of radiolabeled angiotensin II in the adrenal cortex uncovered a regulatory effect of guanine nucleotides on agonist interaction with adrenal receptor sites. Since guanine nucleotide modulation of hormone-receptor interaction is typically observed in adenylate cyclase-coupled receptor systems, this observation initially appeared at variance with the apparent lack of a stimulatory effect of angiotensin II on cyclic AMP production. Similar observations of a regulation by guanine nucleotide of angiotensin II binding in liver (8), where the octapeptide inhibits cyclic AMP production, and of an inhibitory effect of angiotensin II on adenylate cyclase activity in kidney (9), suggest that angiotensin II receptors might be generally coupled to adenylate cyclase in an inhibitory fashion.

In adenylate cyclase-coupled receptor systems, guanine nucleotide regulation of agonist binding is best characterized by performing competition curves of agonists for the binding of radiolabeled antagonists in plasma membrane preparations. Such studies of the characteristics of the direct interaction of agonists and antagonists have generally revealed the existence of an apparent agonist-specific, guanine nucleotide-sensitive, high affinity form of the receptor sites (10). This high affinity form has been shown to involve a tight interaction of the receptor with a guanine nucleotide-binding protein (11). Biochemical evidence for this agonist-induced ternary complex of hormone, receptor, and guanine nucleotide-binding protein has been provided by demonstrating that agonist-occupied solubilized receptors display a larger apparent molecular size than antagonist-occupied solubilized receptors (12–14). The larger size agonist-occupied receptor fraction presumably incorporates one of the guanine nucleotide-binding proteins involved in hormone-mediated stimulation or inhibition of adenylate cyclase activity (15–17).

In order to investigate the differential binding properties of agonists and antagonists to angiotensin II receptor in bovine adrenal cortex, we have developed and used the new radiolabeled antagonist [^{125}I] [Sar^1, Ile^8] angiotensin II in receptor saturation and competition experiments with various analogues of the hormone. Agonist-induced changes in the molecular properties of adrenal cortex angiotensin II receptors were also documented by comparing the properties of agonist- or antagonist-occupied receptors following solubilization with detergent. The results support the hypothesis that agonists stabilize the reversible interaction of the receptor protein with a guanine nucleotide-binding protein.

MATERIALS AND METHODS

Bovine adrenal subcapsular layer membrane preparation. A 0.5-mm subcapsular layer was dissected and homogenized in 20 mM $NaHCO_3$ according to Glossman *et al.* (6). The washed 40,000 × *g* pellet was resuspended in 250 mM sucrose, 50 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 0.1 mM EDTA and stored at −70°. Protein was determined according to the method of Lowry *et al.* (18).

Iodination of peptides. SARILE[3] was labeled with ^{125}I using Iodo-Beads (Pierce Chemical Co.). Carrier-free ^{125}I (2.5 mCi, New England Nuclear) was added to 100 μl of SARILE (1 mg/ml) in a siliconized glass tube. The reaction was started by adding two Iodo-Beads and carried out for 15 min at 4°. Then 200 μl of 0.05 M phosphate, pH 7.4, was added and the reaction mixture was transferred onto a DEAE-Sephadex column (1 × 100 cm) previously equilibrated with 0.05 M phosphate, pH 7.4 at 4°, and with 5 ml of 2% bovine serum albumin in phosphate buffer immediately before applying the iodination reaction mixture. Immunoreactive iodinated peptide was assayed in the eluted fractions (1 ml) using an anti-SARILE antibody from rabbits immunized (19) with SARILE coupled to thyroglobulin (20). Immunoreactive fractions were pooled, fractionated, and stored at −70° for up to 6 weeks. The same procedure was used for iodinating SARANG except that the iodinated peptide was detected with anti-angiotensin II antibody and that carrier-free ^{131}I was often used instead of ^{125}I.

Angiotensin II was iodinated according to the chloramine-T method of Greenwood *et al.* (21) and purified as for SARILE. Specific activity of [^{125}I]ANG II was assessed by using rabbit angiotensin II antibody and ranged from 600 to 800 Ci/mmol. Specific activity of [^{125}I]SARILE was estimated by comparing receptor saturation curves for undiluted ^{125}I-labeled octapeptide and iodinated antagonist diluted 100 times with unlabeled peptide. The ratio of the total binding capacity of pure ^{125}I-antagonist expressed in counts per min divided by the total binding capacity of quenched peptide expressed in mass units (femtomoles) provides an estimate of specific activity ranging from 300 to 500 Ci/mmol.

The effect of iodination on the properties of the hormone were checked by studying the properties of angiotensin II iodinated with the same method but after substitution of Na^{125}I for NAI. The iodinated peptide was tested for its ability to stimulate aldosterone production in bovine adrenal zona glomerulosa cells and for its binding characteristics in competition curves against [^{125}I]ANG II or [^{125}I]SARILE. In all these tests, the iodinated hormone displayed a potency indistinguishable from that for native hormone.

Radioligand binding in intact membranes. [^{125}I]ANG II and [^{125}I] SARILE were incubated for 60 min at 25° in 1 ml containing 300–600 μg of membrane protein, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 0.1 mM EDTA, and 0.2% heat-inactivated bovine serum albumin. The reaction was stopped by filtration on GF/B glass filters followed by four washings with 5 ml of ice-cold incubation buffer.

Prelabeling of membrane angiotensin II receptors. The agonist [^{131}I] SARANG or the antagonist [^{125}I]SARILE were used for labeling angiotensin II receptors prior to solubilization. Adrenal subcapsular membranes were incubated for 30 min at 25° with radiolabeled peptide (10^7 cpm) containing 1.5–6 mg/ml of membrane protein, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA, and 0.2% heat-inactivated bovine serum albumin. The reaction was stopped by dilution with buffer at 4°C and centrifugation for 10 min at 40,000 × *g*. Membranes were washed once with buffer at 4°. This procedure was shown to remove most of the unbound radiolabeled peptide without promoting dissociation of receptor-bound peptide. When simultaneous labeling with agonist and antagonist was required, membranes were split into two batches which were incubated which [^{131}I]SARANG or [^{125}I]SARILE, respectively. Following the first centrifugation and washing, labeled membranes were then pooled and processed together, resulting in a dual labeling of angiotensin II receptors. In some earlier experiments, membrane preparations labeled with [^{125}I]SARILE or [^{125}I]SARANG were kept separate throughout the rest of the experiment.

Solubilization of membrane receptors. Agonist- and/or antagonist-prelabeled membrane receptors were solubilized at a concentration of 2–3 mg of membrane protein/ml of medium containing 10 mM Tris-acetate, pH 8.0, 40 mM octyl-β-D-glucoside, 10 mM $MgCl_2$, 0.1 mM EDTA at 4°. The washed membrane pellet was resuspended in solubilization buffer and gently stirred on ice for 20–30 min. The supernatant was carefully aspirated and used immediately. Centrifugation at

[3] The abbreviations used are SARILE, [Sar^1, Ile^8]angiotensin II; SARANG, [SAR^1]angiotensin II; ANG II, angiotensin II; ANG III, [des-Asp^1]angiotensin II; Gpp(NH)p, guanylylimidodiphosphate; pK,−$\log_{10}$ of K_d; TAO buffer, 10 mM Tris-acetate, pH 8.0, 40 mM octylglucoside, 0.1 mM EDTA.

250,000 × *g* for 60 min and/or filtration through 0.22 μm pore size filters yielded the same results.

Separation of bound and free peptide. Since both radiolabeled peptides slowly dissociate from angiotensin II receptor sites, it was necessary to separate bound from free peptide in order to accurately assess receptor-bound hormone. Aliquots (0.5 ml) of soluble preparation were processed on Sephadex G-50 columns (0.6 × 12 cm) as described by Caron and Lefkowitz (22). The column was first equilibrated with solubilization buffer. Following adsorption of a 0.5-ml aliquot, a void volume fraction of 2 ml was collected. This fraction was shown to contain all blue dextran used as a marker. Free radiolabeled peptide was eluted in the next 4–5 ml.

Size of detergent micelles. In order to monitor the elution profile of detergent micelles, the lipophilic dye Oil Red O was "solubilized" in buffer containing 40 mM octyl-β-D-glucose and excess insoluble dye was removed by ultrafiltration through a 0.22-μm pore size filter (Millipore). When processed in an Amicon ultrafiltration cell, the dye-colored micelles were retained with a PM10 membrane, but passed through a PM30 membrane. A 0.5-ml aliquot of the detergent solution of Oil Red O eluted in the latter 0.5 ml of the void volume (2 ml) on a Sephadex G-50 column (0.6 × 12 cm). Thus, octylglucoside micelles behave as particles with a size between 10,000 and 30,000 Da and are excluded on Sephadex G-50 columns. However, these micelles do not entrap significant amounts of radiolabeled peptides.

Delipidation and initial purification of solubilized receptor on DEAE-agarose. A DEAE-agarose (Bio-Rad) column (1.0 × 3.8 cm; 3-ml bed volume) was equilibrated with TAO buffer. The solubilized preparation (6 ml) was immediately applied to the column at a flow rate of 30 ml hr^{-1}. The column was washed with 9 ml of TAO buffer and then with 9 ml of TAO buffer containing 50 mM sodium acetate. Receptor-bound radiolabeled ligand was then eluted with 9 ml of TAO buffer containing 150 mM sodium acetate. Phospholipids were assayed with ferrothiocyanate (23). The occurrence of receptor-bound peptide in the radioactivity peak fractions was checked by chromatography on Sephadex G-50.

Separation of agonist- and antagonist-occupied receptor by steric exclusion high performance liquid chromatography. The high performance liquid chromatography system consisted of a pump (Waters Associates, Milford, MA, model 6000A), an injection valve (Rheodyne, Berkeley, CA, model 7125) with a 2-ml loop, dual Bio-Sil TSK-250 columns (0.75 × 30 cm) (Bio-Rad, Mississauga, Ontario, Canada), and a fraction collector (Pharmacia, Montreal, Canada, model FRAC100). The mobile phase consisted of TAO buffer containing 150 mM sodium acetate and 0.1% triethylamine. The mobile phase and the columns were maintained at 4° during the chromatography. The flow rate was fixed at 1 ml min^{-1}. A 1–1.5-ml aliquot of receptor-bound radioligand peak eluted from DEAE-agarose was filtered through a 0.22-μm pore size filter (Millipore) before injection. The first 10 ml of the effluent corresponding to the void volume of the columns were discarded; 250-μl fractions from 10 to 30 ml of the effluent were then collected for radioactivity measurement. The calibration of the gel exclusion system was done with the following protein standards: thyroglobulin, catalase, ferritin, aldolase, chymotrypsinogen A, and ribonuclease. The detection was done with a UV variable detector (Waters model 450) at 280 nm.

Primary culture of bovine adrenal subcapsular cells. A 0.5-mm subcapsular layer of adrenal glands from freshly slaughtered cattle was dispersed with collagenase (2 mg/ml) and DNase (0.2 mg/ml), plated (1.5 million cells/ml) in minimum essential medium with 10% horse serum as previously described (24). Cells were stimulated for 3 hr on day 4 after replacement with fresh medium without serum. Aldosterone in the medium was measured by radioimmunoassay with [^{3}H]aldosterone using dextran/charcoal to remove free radioligand (24). Angiotensin II and all other agonists used typically stimulated 10- to 15-fold aldosterone production, indicating that they all behave as full agonists. None of the antagonists used displayed any agonist activity at concentrations below 1 μM. Comparative properties of analogues from bovine adrenal cortex are shown in Table 1.

Computer modeling. Saturation and competition binding curves were analyzed by nonlinear least squares curve fitting according to Marquardt and Levenberg, using the mass-action law model of Feldman for multiple ligands and multiple binding sites (10). Equilibrium constants K (i.e., $1/K_d$) for the binding of peptides to receptor sites are expressed in terms of $-\log_{10}K_d$ or pK. Saturation and competition curves were analyzed according to a model for one or two classes of sites. The more complex model was retained only when it statistically improved the goodness of the fit (10). Competition curves for agonists in the presence and in the absence of guanine nucleotides were analyzed simultaneously. In the case where only one class of sites could be documented for the curve in the presence of guanine nucleotides, but two classes for curve in its absence, the two affinity constants for the curve with Gpp (NH)p where merged together by constraining them to be equal (10). Kinetics of the association and the dissociation of the peptide were analyzed by curve fitting with the exponential function $B_t = B_{eq} \cdot (1 - \epsilon^{-\tau t})$ and $B_t = B_0 \cdot \epsilon^{-\tau t}$ respectively. B_t represents the concentration of peptide bound at time t, B_{eq} and B_0 correspond to the concentration of ligand bound at equilibrium and at the onset of dissociation, while τ is the time constant of the reaction.

Materials. Angiotensin II, [Sar^1, Ile^8]angiotensin II, and [Sar^1, Leu^8] angiotensin II were from Peninsula. [Sar^1]angiotensin II, [des-Asp^1] angiotensin II, and [des-Asp^1,des-Arg^2]angiotensin II were synthesized by the solid phase method according to a protocol published elsewhere (25). Peptides were purified by partition chromatography on Sephadex G-25 with the system 1-butanol/acetic acid/H_2O (4:1:5) and by reversed-phase chromatography on an octadecasilylsilica column using linear gradients of methanol in 1% trifluoroacetic acid. Purity of the peptides was established by high performance liquid chromatography, thin layer chromatography, and amino acid analysis. Carrier-free ^{125}I and ^{131}I were purchased from New England Nuclear. Sources of media, serum, and enzymes for cell culture were as reported (24). Octyl-β-D-glucoside was from Calbiochem. Protein calibration standards were from Pharmacia. Phosphatidylcholine was from Sigma.

RESULTS

[^{125}I]SARILE binding to adrenal subcapsular membrane receptor sites is fast and reversible (Fig. 1). At a low concentration (0.4 mM), binding of the ^{125}I-antagonist reaches steady state conditions after 60 min of incubation. Receptor bound ^{125}I-antagonist is stable at least for 120 min. Dissociation of bound ^{125}I-peptide promoted by addition of an excess of native antagonist proceeds slowly with a time constant of 4.7×10^{-3} min^{-1}. This rate of dissociation of [^{125}I]SARILE is 1 order of magnitude slower than that reported by Glossman *et al.* (6) for [^{125}I]ANG II under the same experimental conditions. Nonspecific binding of the antagonist in the constant presence of an excess of the peptide is very low and does not vary within 2 hr.

Figure 2 shows a typical example of a receptor saturation curve with [^{125}I]SARILE. The binding curves in the absence and in the presence of excess unlabeled antagonist were best fit to a model for a single class of receptor-binding sites with a pK of 9.0. Thus, the antagonist appears to react with the same affinity to a homogeneous population of binding sites.

Previous studies with radiolabeled angiotensin II suggested two binding components in adrenal subcapsular tissue (6). Since the results in Fig. 2 for [^{125}I]SARILE indicated a single class of receptor sites, we next performed receptor saturation curves with ^{125}I-agonist under the same conditions as those for the antagonist. The results confirmed the more complex nature of agonist

TABLE 1

Comparative properties of agonist and antagonist analogs of angiotensin II in bovine adrenal cortex

	Ang II	[Sar¹]Ang II	Ang III	[des-Arg¹] Ang III	[Sar¹,Ile⁸] Ang II	[Sar¹,Leu⁸] Ang II
−Log potency[a]	9.2 ± 0.04 (2)	10 ± 0.1 (2)	7.4 ± 0.2 (3)	5.0 ± 0.07 (4)	8.9 ± 0.2 (2)	9.1 ± 0.1 (2)
Activity	Agonist	Agonist	Agonist	Agonist	Antagonist	Antagonist
pK_H[b]	8.8 ± 0.2 (2)	9.6 ± 0.2 (2)	9.2 ± 0.1 (3)	5.9 ± 0.1 (4)		
					8.9 ± 0.1 (2)[c]	9.1 ± 0.1 (2)
pK_L	7.6 ± 0.03 (2)	8.5 ± 0.1 (2)	7.6 ± 0.1 (3)	4.1 ± 0.1 (4)		
K_H/K_L[d]	14 ± 3 (2)	13 ± 3 (2)	40 ± 6 (3)	63 ± 9 (4)	1	1
$\%R_H$						
−Gpp(NH)p[e]	60 ± 2 (2)	56 ± 6 (2)	42 ± 2 (3)	18 ± 3 (4)		
+Gpp(NH)p[e]	12 ± 12 (2)	16 ± 16 (2)	0 ± 0 (3)	0 ± 0 (4)		
Slope factor						
−Gpp(NH)p[f]	0.08 ± 0.04 (2)	0.88 ± 0.02 (2)	0.70 ± 0.02 (3)	0.80 ± 0.05 (4)	0.96 ± 0.04 (2)	0.98 ± 0.04 (2)
+Gpp(NH)p[f]	0.89 ± 0.04 (2)	0.92 ± 0.01 (2)	0.91 ± 0.03 (3)	1.02 ± 0.04 (4)	0.96 ± 0.04 (2)	0.98 ± 0.02 (2)

[a] −Log potency on aldosterone production in cultured bovine adrenal subcapsular cells.
[b] Log of equilibrium constant.
[c] Receptor sites displayed homogeneous affinity for antagonists.
[d] Radio of equilibrium constants for low and high affinity forms.
[e] Relative proportion of high affinity form with or without Gpp(NH)p.
[f] Slope factor of competitive binding curves with or without Gpp(NH)p.

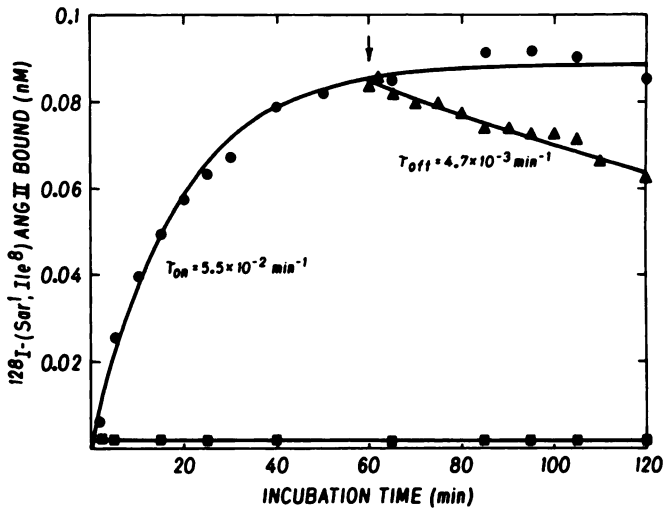

FIG. 1. *Time course of association and dissociation of 0.38 nM [^{125}I] SARILE at 25°*

The difference between total binding (●) and nonspecific binding in the constant presence of 1 μM unlabeled SARILE (■) represents specific binding. After 60 min of association, a small volume of SARILE was added to attain a final concentration of 1 μM in order to promote dissociation of the ligand (▲). The time constants for association (τ_{on}) and dissociation (τ_{off}) of peptide were estimated by nonlinear least squares curve fitting with monoexponential equations.

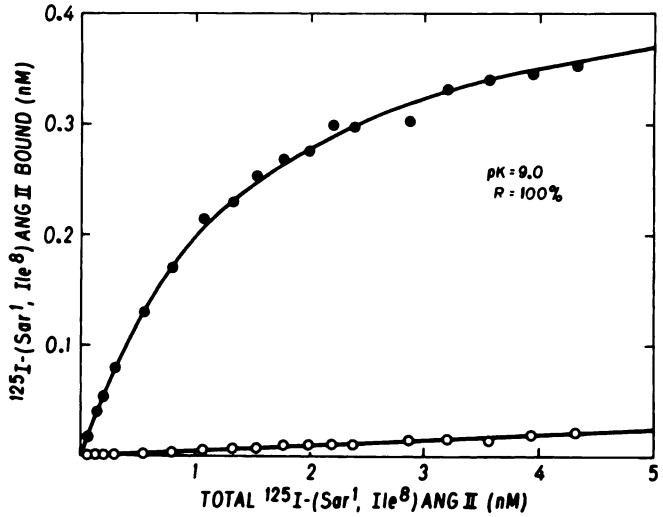

FIG. 2. *Receptor saturation curve for the angiotensin [^{125}I]SARILE*

[^{125}I]SARILE was incubated with 315 μg of protein of membrane preparation in the absence (●) or in the presence (○) of 10^{-6} M angiotensin II. Computer modeling of specific binding calculated as the difference between total (●) and nonspecific (○) binding was best explained by a single population of sites with pK 9.

binding to adrenal cortex receptor sites. Figure 3 shows that the saturation curve for [^{125}I]ANG II is best explained by nearly equal proportions of two binding components with pK of 8.8 and 7.6, respectively. These estimates of the affinity of the hormone for the two binding components closely compare with those (pK 8.7 and 7.5) reported earlier by Glossmann *et al.* (6). Thus, it appears that the antagonist [^{125}I]SARILE uniformly binds to all angiotensin II receptor sites and that the agonist [^{125}I]ANG II discriminates among these sites a high and a low affinity component.

A more extensive study of the differential properties of agonist and antagonist analogues of angiotensin II was performed with competitive binding studies using a series of agonists and antagonists. Figure 4 shows that a competition curve of unlabeled SARILE for the binding of [^{125}I]SARILE is best explained by a homogeneous population of binding sites having equal affinity for the competing antagonist. The curves obtained in the presence or the absence of guanine nucleotide are superimposable and are characterized by a slope factor of 1 and a single pK of 8.8. These results confirm the observation obtained with the saturation curve for [^{125}I]SARILE (Fig. 2). They also indicate that the unlabeled and labeled forms of the antagonist SARILE have similar properties in receptor-binding assays. Figure 4 also documents that

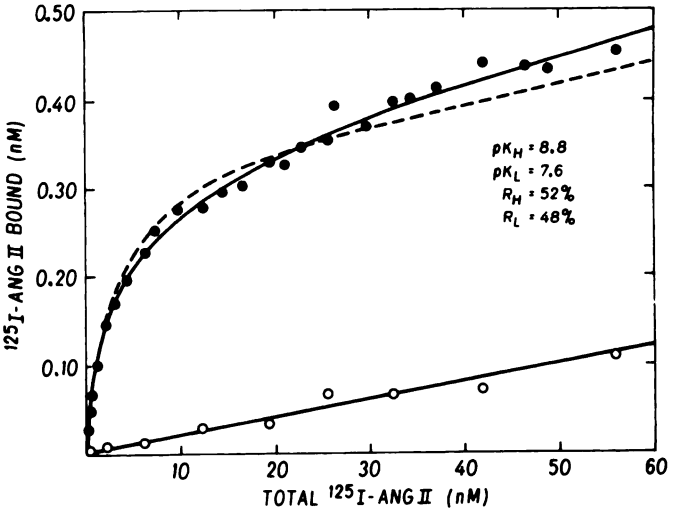

FIG. 3. *Receptor saturation curve for the agonist [^{125}I]angiotensin II.* [^{125}I]angiotensin II was diluted to 3.2 Ci/mmol with unlabeled hormone and incubated in 1 ml with 320 μg of membrane protein. The difference between total (●) and nonspecific (○) binding obtained in the absence and the presence of 10^{-6} M angiotensin II was significantly better explained ($p = 0.03$) by a model for two binding components (— —) than by a model for a single binding component (- - -). Parameters pK_H and pK_L are the logarithms of equilibrium constants of the high and the low affinity components, while R_H and R_L are their relative proportions.

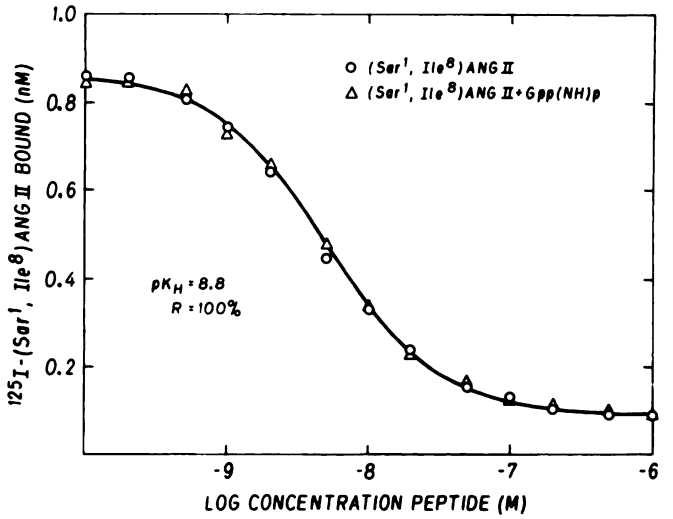

FIG. 4. *Competition curve of the antagonist SARILE for the binding of [^{125}I]SARILE (4.2 nM) in 1 ml containing 640 μg of membrane protein with (△) or without (○) 10^{-4} M GPP(NH)p*

Computer modeling indicated that the curve had a normal slope factor of 1 and was best explained by a single homogeneous population of binding sites with pK 8.8 corresponding to the value obtained in Fig. 2 for a saturation curve with the same peptide. No effect of Gpp(NH)p could be detected.

[^{125}I]SARILE does not display any sensitivity to a guanine nucleotide.

In contrast, a competition curve with the agonist angiotensin II is more complex and sensitive to addition of a guanine nucleotide (Fig. 5). Computer analysis of the competition curve without guanine nucleotide provides estimates of a proportion of 62% high affinity binding component of the receptor with pK of 8.6, with the

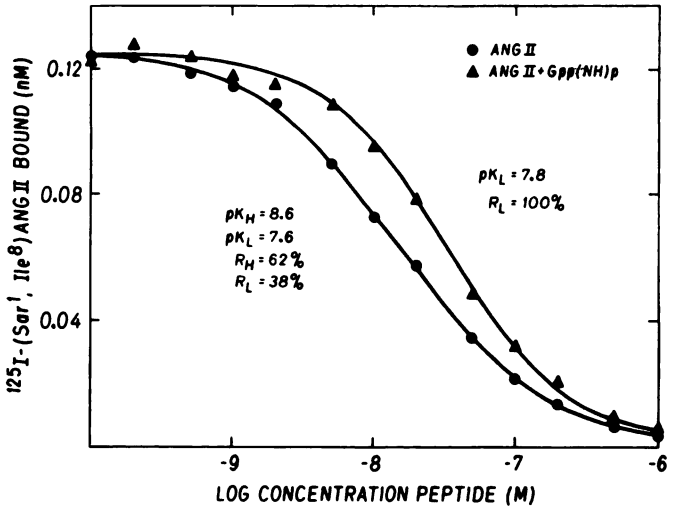

FIG. 5. *Competition curve of angiotensin II for the binding of [^{125}I] SARILE (0.26 nM) in 1 ml containing 560 μg of membrane protein with (▲) or without (●) 10^{-4} M Gpp(NH)p*

Addition of the guanine nucleotide increased the slope factor of the competition curve from 0.84 to 0.92. Computer modeling indicated that in the absence of guanine nucleotide the curve was best explained ($p < 0.001$) by a mixture of 62% high affinity and 38% low affinity binding component with pK 8.6 and 7.6, respectively, in agreement with the results shown in Fig. 3 for a saturation curve with the same peptide. In the presence of guanine nucleotide, only one component was documented, whose affinity (pK 7.8) is not statistically different from that of the lower affinity component observed in the absence of guanine nucleotide.

remaining binding component having a lower pK of 7.6. Addition of guanine nucleotide increases the slope factor of the curve from 0.84 to 0.92 and decreases the proportion of the high affinity component to a low level, while the remaining sites display an affinity for angiotensin II (pK 7.8) comparable to that for the low affinity form of the receptor in the absence of guanine nucleotide. The estimates of the affinity and proportion of adrenal cortex receptor components documented with unlabeled angiotensin II (Fig. 5) and with its iodinated form (Fig. 3) are again strikingly similar. An even more dramatic effect of Gpp(NH)p on the binding characteristics of agonists is documented in fig. 6 with angiotensin III. The pronounced shift to the right and steepening of the competition curve is consistent with a large difference in the affinity of the two binding components of the receptor with pK values of 9.1 and 7.6 respectively. Interestingly, the proportion of high affinity form in the presence of angiotensin III is lower than that for angiotensin II (45 versus 62%).

In order to assess further the specificity of these binding characteristics, competition curves of various analogs for the binding of [^{125}I]SARILE were obtained and analyzed. All the agonists used displayed full intrinsic activity relative to angiotensin II, when tested for their ability to stimulate aldosterone production *in vitro*. The antagonists used fail to display any agonist activity at concentrations below 1 μM. All the agonists tested display analogous binding characteristics: competition curves with a low slope factor in the absence of guanine nucleotides document two binding components whose propor-

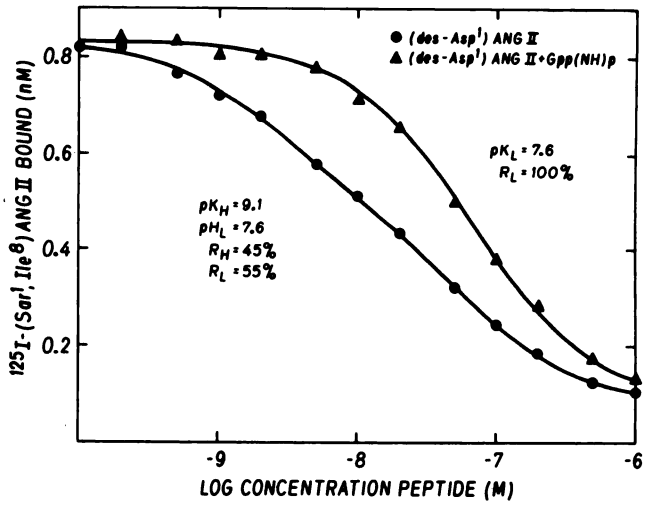

FIG. 6. *Competition curve of angiotensin III for the binding of [^{125}I] SARILE (4.0 nM) in 1 ml containing 600 μg of membrane protein with (▲) or without (●) 10^{-4} M Gpp(NH)*

Addition of the guanine nucleotide increased the slope factor of the competition curve from 0.70 to 0.93. Computer modeling indicated that in the absence of guanine nucleotide the curve was best explained ($p < 0.001$) by a mixture of 45% high affinity and 55% low affinity binding components with pK 9.1 and 7.6, respectively. In the presence of guanine nucleotide, only a low affinity component (pK 7.6) was documented.

tions are modulated by addition of guanine nucleotide. In contrast, antagonists display homogeneous affinity for all binding sites and no sensitivity to the addition of guanine nucleotide. The ratio of equilibrium constants varies from 13 for [Sar^1]ANG II to 63 for [des-Arg^1]ANG III while the proportion of the high affinity component of the receptor varies from 60% for ANG II to 18% for [des-Arg^1]ANG III. Such changes with varying agonists in the proportion of two binding components of the receptor strongly suggest that these components are not independent but rather interconvertible. The potency order of agonists competing for [^{125}I]SARILE binding [Sar^1]ANG II > ANG II > ANG III > [des-Arg^1]ANG III, is the same as the potency order of agonists on aldosterone production. This indicates that [^{125}I]SARILE identifies the binding sites associated with the pharmacological response to angiotensin II.

In order to further document the agonist-specific high affinity form of angiotensin II receptor, we proceeded to the solubilization of radiolabeled agonist- and antagonist-occupied receptor. Among the various detergents tried during the initial attempts at solubilizing angiotensin II receptor, octyl-β-D-glucoside was selected because of its well defined pure synthetic form, its high critical micellar concentration allowing for easy removal by dialysis, its ability to preserve protein-protein interactions, its nonionic nature, and its successful application to the solubilization of other receptors from the same tissue (26). Initial tests on the effect of varying the concentration of detergent on the solubilization of protein-bound [^{125}I]SARILE indicated that concentrations of detergent above critical micellar concentration (25 mM) were required and that optimum solubilization was achieved at 40 mM (data not shown). These results are similar to those reported for the solubilization of the low density lipoprotein receptors (26). About 40–50% of receptor-bound antagonist in the membranes was found associated with proteins in the solubilized preparation. Only 7 to 12% of membrane-bound peptide dissociated from the receptor sites during solubilization.

Agonist binding was typically more susceptible to dissociation during solubilization with detergent. Since we aimed at comparing antagonist-liganded with agonist-liganded solubilized angiotensin II receptor, we attempted to stabilize agonist binding with divalent cations during solubilization. Table 2 shows that addition of magnesium salt (10 mM $MgCl_2$) to solubilization medium stabilizes 20–30% of receptor-bound [^{125}I]SARANG. Higher concentrations of $MgCl_2$ up to 25 mM only slightly improved agonist-binding stability (not shown). Attempts to vary the pH during solubilization indicated that, at pH 6, agonist-bound angiotensin II receptor is exceedingly unstable (not shown), suggesting an important role of ionic interactions in hormone binding to angotensin II receptors.

We then checked whether the selective modulation by guanine nucleotides of agonist binding would be preserved during solubilization. Figure 7 shows that the antagonist [^{125}I]SARILE very slowly dissociates from solubilized angiotensin II receptors and displays no sensitivity towards guanine nucleotide. These results are identical to those observed with membrane preparations and indicate that [^{125}I]SARILE could be used for reversible labeling conformationally intact, solubilized angiotensin II receptors. In contrast to the antagonist analogue, the potent agonist [^{125}I]SARANG dissociates more rapidly from solubilized receptors. Guanine nucleotides enhance agonist dissociation from soluble receptors. These properties again replicate in a solubilized preparation the observations reported from intact membranes (Figs. 4–6). A dissociation rate of the agonist [^{125}I]SARANG apparently faster than that of the antagonist, both in the presence and the absence of guanine nucleotide, suggests that the faster decaying high affinity agonist-receptor complex might involve additional interactions with another protein. This ternary complex might be more susceptible to detergent than the simpler binary complex of the antagonist with the receptor.

TABLE 2

Effect of magnesium on solubilization of [^{125}I]SARANG-bound angiotensin II receptor

Adrenal subcapsular membranes (60 mg) incubated with 1.5 million cpm of [^{125}I]SARANG retained 41% of radiolabeled hormone after washing at 4°. Washed membranes were solubilized at a concentration of 12 mg of protein/ml in 40 mM octylglucoside, 50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, and the indicated concentration of $MgCl_2$. Bound hormone and free dissociated hormone were separated on Sephadex G-50 columns (0.6 × 12 cm).

$MgCl_2$	Bound hormone solubilized	Dissociated hormone
(m_m)	% membrane bound	% membrane bound
0	9	53
2	13	60
5	21	55
10	27	57

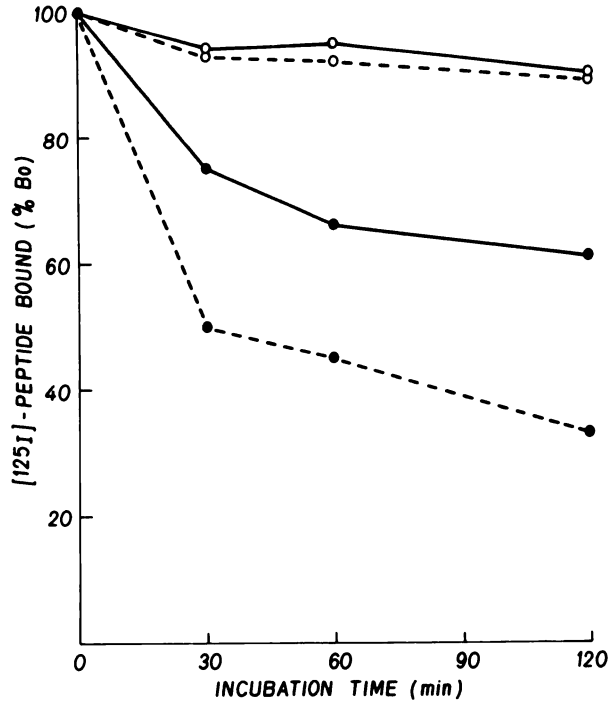

FIG. 7. *Dissociation of agonist [^{125}I]SARANG and antagonist [^{125}I]SARILE from solubilized angiotensin II receptors*

Adrenal subcapsular membranes labeled with either [^{125}I]SARANG or [^{125}I]SARILE were solubilized at a concentration of 15 mg of protein/ml of 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 40 mM octylglucoside, 0.1 mM EDTA at 4°. Timed aliquots (0.5 ml) were processed on Sephadex G-50) columns (0.6 × 12 cm). Radiolabeled peptide bound to solubilized receptor sites immediately after solubilization (B_o) amounted to 33 and 12% of membrane-bound [^{125}I]SARILE and [^{125}I]SARANG, respectively. Dissociation of [^{125}I]SARILE (○) and [^{125}I]SARANG (●) was observed following 5-fold dilution in buffer containing 100 μM Gpp(NH)p (– – –) or no nucleotide (——).

Monovalent cations are well known regulators of agonist binding to many guanine nucleotide-sensitive receptors. Since their use is required in order to increase the ionic strength during ion exchange chromatography, we tested the effect of sodium acetate on [^{125}I]SARILE and [^{131}I]SARANG binding to solubilized receptors. Table 3 shows that increasing concentrations of sodium acetate from 0 to 300 mM is virtually without effect on antagonist [^{125}I]SARILE binding during at least 2 hr. In contrast, increasing ionic strength significantly enhances [^{131}I]SARANG dissociation from solubilized receptor during the same incubation period. These results suggest that ion exchange chromatography of solubilized angiotensin II receptor is likely to lead to a relatively larger loss of agonist binding. These observations do not, however, imply a specific regulatory function of sodium on angiotensin II receptor properties, but simply document the effect of ionic strength on the solubilized receptor.

Bile salts are known to disrupt protein-protein interactions. Sodium cholate has been used for solubilizing guanine nucleotide-binding proteins associated with receptor or adenylate cyclase catalytic proteins (15–17). These ionic detergents can be used to test the requirement of protein-protein interactions for agonist binding. Addition of bile salts together with octyl-β-D-glucoside

TABLE 3

Effect of ionic strength on hormone binding to solubilized angiotensin II receptors

Adrenal subcapsular membranes were separately prelabeled with [^{125}I]SARILE or [^{131}I]SARANG. Washed membranes were pooled and solubilized in 10 mM Tris-acetate, pH 8.0, 10 mM $MgCl_2$, 0.1 mM EDTA, 40 mM octylglucoside. Sodium acetate was then adjusted to the indicated concentration and the fraction of bound radiolabeled peptide was determined by separation on Sephadex G-50 columns (0.6 × 1.2 cm). Results are expressed as percentage of total radiolabeled hormone in the soluble preparation.

Time after solubilization	Sodium acetate	[^{125}I]SARILE bound	[^{131}I]SARANG bound
min	*mM*	% in soluble-fraction	
0	0	67	29
60	0	63	23
	50	63	20
	150	61	17
	300	62	17
120	0	62	19
	50	60	16
	150	57	11
	300	56	8

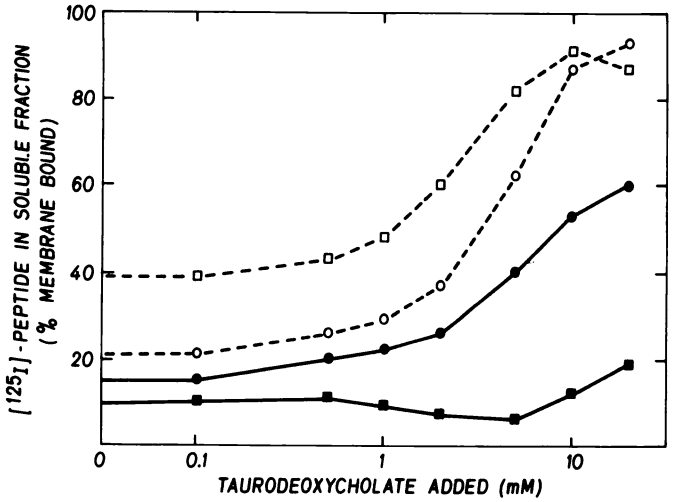

FIG. 8. *Effect of adding bile salt detergent to solubilization of angiotensin II receptor*

Adrenal subcapsular membranes were prelabeled with either [^{125}I]SARILE (○, ●) or [^{125}I]SARANG (□, ■). Washed membranes were then solubilized at a concentration of 4 mg of protein/ml in 10 mM Tris-acetate, pH 7.4, 0.1 mM EDTA, 40 mM octylglucoside, and the indicated concentration of taurodeoxycholate. The results are expressed as percentage of hormone bound to membrane receptor prior to solubilization. The percentage of membrane-bound radioligand recovered in the solubilized preparation (– – –) were measured in the supernatant following centrifugation at 40,000 × *g* for 30 min. Of this total amount of radioligand in the solubilized preparation, protein-bound (——) radioligand was then separated on Sephadex G-50) columns (0.6 × 1.2 cm) The difference between total (– – –) and protein-bound (——) radioligand corresponds to the amount of peptide dissociated from the receptor during the solubilization step.

is expected to lead to differential effects on agonist- and antagonist-receptor complexes if agonist binding involves the interaction of the receptor with a guanine nucleotide-binding protein. Figure 8 shows that tauro-

deoxycholate enhances the solubilization of antagonist-occupied receptor sites from the membrane without promoting dissociation of the ligand. In contrast, addition of the bile salt at a concentration approximating its critical micellar concentration (3 mM) leads to substantial dissociation of the agonist and to a decrease in the solubilized agonist-receptor complex. Thus, dissociation of the agonist [^{125}I]SARANG from soluble receptor is selectively enhanced by the bile salt. These results imply that bile salts destabilize protein-protein interactions between the hormone receptor protein and some other protein required for agonist, but not for antagonist binding.

A more direct demonstration of the involvement of a guanine nucleotide-binding protein in the agonist-specific high affinity form of the angiotensin II receptor involves the comparison of the apparent size of agonist-bound and antagonist-bound solubilized receptor complexes. The agonist-occupied receptor protein is expected to behave as a larger protein than the antagonist-occupied receptor protein, the difference presumably reflecting the size of the guanine nucleotide-binding protein. Initial attempts to demonstrate this property for angiotensin II receptors indicated that a preliminary purification step on DEAE-agarose was required. At low ionic strength, receptor-bound radiolabeled peptides are retained on DEAE-agarose, while free peptides and phospholipids are passing through (Fig. 9). Increasing sodium acetate concentration to 50 mM leads to the elution of a minor component of receptor-bound agonist and antagonist together with heterogeneous proteins loosely retained on the gel. The major fraction of receptor-bound agonist and antagonist is eluted with 150 mM sodium acetate, together with 30–40% of the total protein applied to the gel.

The peak fractions eluted from DEAE-agarose with 150 mM sodium acetate are then fractionated by steric exclusion high performance liquid chromatography. Figure 10 shows typical elution profiles of the agonist [^{131}I]SARANG and the antagonist [^{125}I]SARILE. The receptor-specific peak for the agonist [^{131}I]SARANG eluting between ferritine and catalase displays a distinctly smaller retention volume (14.5 ml) than that for the antagonist [^{125}I]SARILE (16 ml) which eluted between catalase and aldolase. This statistical difference (nine experiments, paired t test = 7.4, $p < 0.001$) would suggest that the receptor behaves as a larger molecular size complex when it is liganded with the agonist. Since it could be hypothesized that these peaks might be due to suboptimal solubilization of membrane proteins, we tested in a separate series of experiments the effect of higher concentrations of detergent. Increasing the concentration of detergent up to 80 mM did not alter the shape of the elution profile (data not shown). Fig. 10 (bottom panel) shows that addition of guanine nucleotide selectively reduces the agonist-receptor complex without altering the antagonist-receptor complex. The effect of Gpp(NH)p on [^{131}I]SARANG is, however, not completely abolished (Fig. 10, bottom panel). This incomplete reduction of the receptor-specific peak for the agonist is reminiscent of a residual high affinity component of membrane angiotensin II receptor with Gpp(NH)p (Table 1). Similar observations of an incomplete transition to low affinity binding has also been reported in other systems (27, 28). The significance of this residual form of high affinity agonist binding which is apparently insensitive to guanine nucleotides is currently being studied. Figure 10 also demonstrates that Gpp(NH)p does not alter the elution profile of the antagonist [^{125}I]SARILE. This further substantiates the insensitivity of [^{125}I]SARILE binding documented in Table 1 and Figs. 4 and 7.

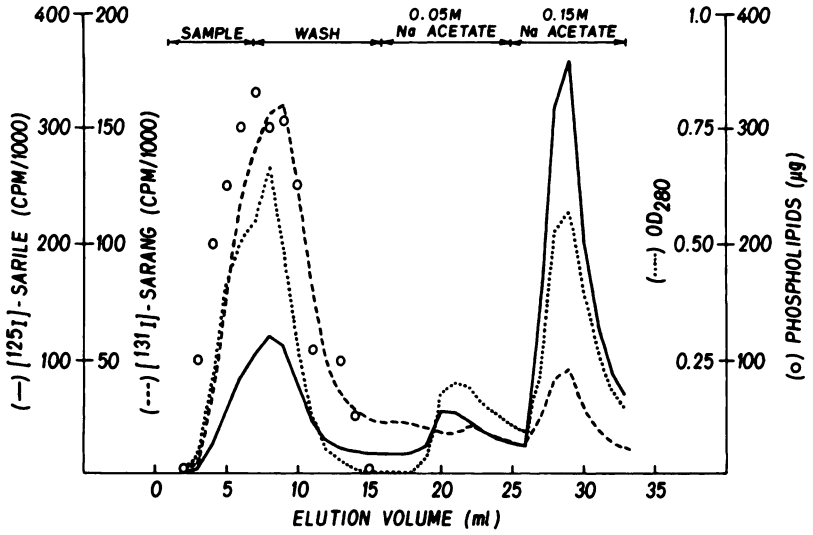

FIG. 9. *Elution profile of angiotensin II receptor on DEAE-agarose*

Two membrane fractions were separately labeled with [^{125}I]SARILE or [^{131}I]SARANG. Washed membranes were pooled and solubilized together in 6 ml of TAO buffer supplemented with 10 mM $MgCl_2$. The 40,000 × g supernatant was applied onto DEAE-agarose (1.0 × 3.8 cm) and washed with TAO buffer supplemented with 50 mM sodium acetate. Receptor-bound hormone was eluted with TAO buffer containing 150 mM sodium acetate. This fraction contains 57% [^{125}I]SARILE (——), 15% [^{131}I]SARANG (– – –), 37% protein (. . .)0, and 0% phospholipids (O) eluted. Actual data points for 1-ml fractions are shown connected by solid, dashed, or dotted lines, except for the elution profile of phospholipids.

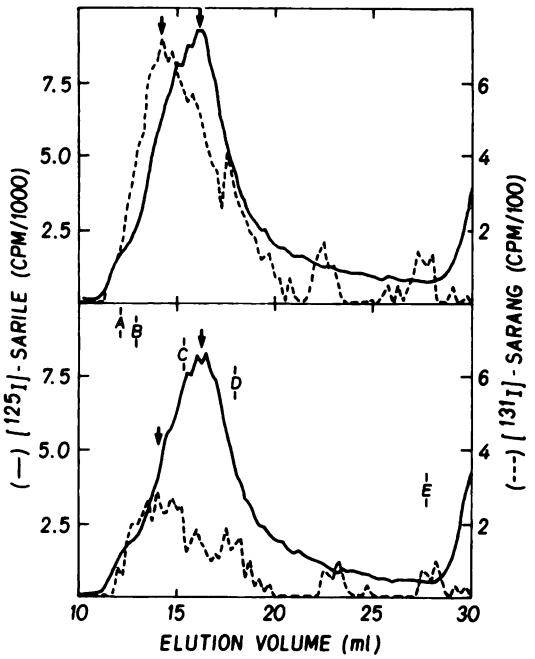

FIG. 10. *Effect of guanine nucleotide on hormone-occupied solubilized angiotensin II receptor*

Adrenal subcapsular membranes were prelabeled with either [^{125}I] SARILE or [^{131}I]SARANG, then washed, and pooled. Prelabeling was performed either in the absence (upper panel) or in the presence (lower panel) of 100 μM guanosine-5′-*O*-(3-thiotriphosphate). Solubilized samples were first processed on DEAE-agarose, and then chromatographed on dual Bio-Sil TSK-250 columns (0.75 × 30 cm). Actual data points for 0.25-ml fractions are shown connected with solid or dashed lines. The arrows indicate the receptor-specific agonist and antagonist peaks. Standards used were: *A*, thyroglobulin (669,000 Da); *B*, ferritin (440,000 Da); *C*, catalase (232,000 Da); *D*, aldolase (158,000 Da); *E*, ribonuclease (13,700 Da).

To document the specificity of the eluted peaks, addition of 10 μM angiotensin II during membrane labeling prior to solubilization abolishes the antagonist peak, indicating that it corresponds to a complex of ^{125}I-antagonist bound to specific angiotensin II receptors (data not shown). The main peak for [^{131}I]SARANG corresponds to specific binding to angiotensin receptor since it is also abolished by addition of 10 μM angiotensin II during membrane labeling (data not shown). Accessory peaks for [^{131}I]SARANG eluting close to and after aldolase correspond to nonspecific binding since they are observed even after adding 10 μM angiotensin II during membrane prelabeling.

DISCUSSION

We have reported the use of the new radiolabeled antagonist [^{125}I]SARILE for the direct identification of adrenal cortex angiotensin II receptor sites. The radiolabeled peptide, which displays no agonist activity in this system, uniformly interacts with these receptor sites with high affinity, stability, and a slow dissociation rate. In contrast with [^{125}I]ANG II which binds to two apparent classes of sites and which is sensitive to the presence of guanine nucleotides, [^{125}I]SARILE displays homogeneous affinity for all the receptor sites to which angiotensin is binding and is insensitive to guanine nucleotides. In agreement with the results reported by Crane *et al.* (8), our preliminary studies with the antagonist [^{125}I]saralasine revealed that this other radioligand demonstrates some residual sensitivity towards guanine nucleotides (data not shown). [^{125}I]Saralasine also had a significantly lower affinity for angiotensin II receptor sites. Thus, [^{125}I]SARILE offers many advantages as a radioligand for angiotensin II receptors.

We have used [^{125}I]SARILE in competitive binding studies with various agonists and antagonists in order to study their distinctive receptor-binding properties. These studies first reported here for this receptor system revealed that all agonists tested share the same characteristics, i.e., discrimination of varying proportions of high and low affinity binding components of the receptor. Guanine nucleotides induced an interconversion of the high affinity form into a low affinity form of the receptor. These properties were not observed with antagonists which evenly interacted with all receptor-binding sites. Examination of structure-activity relationships indicates that the structural requirements for these receptor-binding properties are the same as those for the biological activity of the peptides. The C-terminal portion (Phe^8) is crucial both for hormonal activity and for discrimination of the high affinity form of the binding sites. Alterations of the N-terminal portion (Asp^1 and Arg^2), only lead to changes of potency or affinity of the peptide for the receptor. The striking similarity of the structural requirements for intrinsic activity and binding properties strongly suggests that agonist-specific high and low affinity binding components of angiotensin II receptors reflect, as they do in other systems (10, 11), early molecular events taking place during hormone-induced receptor activation.

In their studies on hepatic angiotensin receptor, Crane *et al.* (8) also documented two binding components in saturation curves for the agonist [^{125}I]ANG II, but only one component in saturation curves for the antagonist [^{125}I]saralasin. Guanine nucleotide selectively reduced the number of agonist-specific high affinity component. Our results obtained in competition curves with agonists and antagonists confirm and extend their report on differences between the receptor-binding characteristics of [^{125}I]angiotensin and [^{125}I]saralasin. Selection of the guanine nucleotide-insensitive antagonist [^{125}I]SARILE for competition curves with a series of agonists leads to the necessary conditions for demonstrating the applicability to this system of our model for two interconvertible forms of a receptor (10, 11). Wright *et al.* (29) have also demonstrated two classes of sites for myocardial angiotensin receptor. However, their interpretation of competition curves for the binding of [^{125}I]angiotensin II suggested that antagonists selectively interacted with the high affinity form of the receptor observed in the presence of agonists. We failed to detect any difference in the relative ability of agonists and antagonists to compete maximally for the binding of [^{125}I]SARILE. Moreover, the total number of receptor sites measured with the agonist [^{125}I]ANG II or the antagonist [^{125}I]SARILE were not different, indicating that agonists and antagonists interact with the same total number of angiotensin

receptor in the adrenal cortex. Wright *et al.* (29) also failed to document reciprocal changes in the number of high affinity and low affinity sites in their analysis of competition curves of angiotensin II for the binding of [^{125}I]angiotensin. The inaccuracy of the binding parameters which they reported for the low affinity binding sites precludes, however, any definitive conclusion about the applicability of a model for two interconvertible forms to the myocardial angiotensin receptor.

The observation of two binding components of ANG II receptor sites would be compatible with several alternative models previously considered for other systems (11). These binding components might correspond to two independent classes of sites. This latter model would, however, be incompatible with the observation of changing proportions of high and low affinity binding components with varying agonists or with addition of guanine nucleotides (Table 1). The dimeric receptor model, compatible with the early biochemical characterization by Capponi and Catt (30) of the ANG II receptor protein, would be an attractive alternative. This latter model, however, predicts constant proportions of the two apparent binding components of the receptor (11), again in contradiction to the observation of varying proportions of the two forms of the receptor. A model involving agonist-induced interaction of the receptor with an another membrane protein cannot be ruled out (11). This ternary complex model has been suggested for various adenylate cyclase-coupled receptor systems (10, 11, 13, 27, 28). This proposed mechanism of interaction of the receptor with a regulatory protein is further buttressed by our first demonstration of differential properties of agonist and antagonist binding to solubilized angiotensin II receptor.

Reversible labeling of conformationally intact forms of solubilized receptors required high affinity ligands with slow dissociation rates in order to preserve a high level of radioactive signal throughout the experiment. The potent full agonist SARANG proved to be appropriate, although its rate of dissociation from solubilized receptors is faster than that for the antagonist SARILE. Then, because of its stable interaction with angiotensin receptor during solubilization, SARILE constitutes a potentially useful reversible marker of conformationally active angiotensin II receptor protein during its partial purification. This approach might advantageously compare with other methods involving covalent attachment of photoreactive analogues of angiotensin II. Simultaneous labeling of angiotensin II receptors that we first report using [^{131}I]SARANG and [^{125}I]SARILE allows for a direct comparison of the apparent size of agonist-occupied and antagonist-occupied receptor complexes in the same chromatographic sample. This double labeling technique eliminates the potential influence of fluctuations during chromatography.

The highly reproducible difference in the retention volume of agonist and antagonist peaks might be attributed to a major conformational change or to dimerization of the receptor. However, these hypotheses would fail to explain the specific effect of guanine nucleotides on receptor-bound [^{125}I]SARANG. Our preferred hypothesis is that the increase in the apparent size of the receptor is due to its interaction with a guanine nucleotide-binding protein (15–17). The same observation of agonist-induced increase in the apparent size of *beta*-adrenergic, *alpha*-adrenergic, and dopamine receptors has been similarly interpreted (12–14).

The identity of the final membrane effector(s) of adrenal cortex angiotensin II receptors is still obscure. Adenylate cyclase activity is inhibited by angiotensin II (5). However, this inhibition is likely to play a permissive or modulatory role rather than a primary role in the steroidogenic effect of angiotensin II on the adrenal cortex. A plausible alternative suggested by Peach (1) involves coupling of the angiotensin II receptor, through a nucleotide-binding protein, but with an effector distinct from the catalytic subunit of adenylate cyclase, perhaps in connection with phosphatidylinositol metabolism. Agonist-induced interaction of angiotensin II receptor with another membrane protein regulating its properties might represent one of the important early events during receptor activation by the octapeptidic hormone. Further insight in the molecular mechanism of this hormone is awaiting the identification of the other components of this receptor system.

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