JCI The Journal of Clinical Investigation

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J Clin Invest. 1977;59(1):69-81. https://doi.org/10.1172/JCI108623.

Research Article

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High Affinity Binding of ^{125}I-Angiotensin II to Rat Glomerular Basement Membranes

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ABSTRACT ^{125}I-angiotensin II (AII) specifically bound to rat glomerular basement membrane (GBM). The kinetics of binding were similar to those obtained with the total glomeruli. The apparent dissociation constant was close to 50 pM with both preparations. The number of sites related to the amount of protein was two times greater with GBM than with total glomeruli. Since the amount of GBM protein extracted from a given amount of glomerular protein was about 10%, it was possible to estimate the share of the GBM binding sites for AII as representing 20% of the total number present in the entire glomerulus. Binding studies at equilibrium as a function of ^{125}I-AII concentration and competitive binding experiments suggested either multiplicity of the binding sites or cooperativity in the binding reaction. Degradation of ^{125}I-AII in the presence of GBM was slight and did not increase with time. The difference in the degrees of degradation of ^{125}I-AII was too small to account for the observed difference in binding when the results obtained with GBM and isolated glomeruli preparations were compared. ^{125}I-AII binding to GBM was increased after treatment of these membranes with collagenase, slightly diminished with neuraminidase, and almost completely abolished with trypsin suggesting the proteic nature of the receptor. ^{125}I-AII binding to GBM was diminished after incubation of GBM with anti-GBM antibodies as a result of a decrease in the number of binding sites. ^{125}I-AII binding was even more diminished in preparations of glomeruli isolated from rats passively immunized with anti-GBM antibodies when compared with glomeruli from control animals. This resulted from both smaller affinity for AII and decrease in the number of the binding sites. The present data provides evidence for specific binding sites for AII localized on GBM. This is noteworthy since receptors for polypeptide hormones are currently observed on the surface of cell membranes. These findings also suggest a new physiological role for AII which might involve modification of GBM permeability.

Received for publication 24 March 1976 and in revised form 27 September 1976.

INTRODUCTION

It has been clearly shown that Angiotensin II (AII)[1] labeled either with ^{125}I- (1, 2) or [^{3}H] (2) binds specifically to glomeruli obtained from rat kidney cortex. Furthermore the multiplateau-curve obtained when specific binding is plotted against the concentration of AII in the medium (2) suggests that several groups of binding sites are probably present. Osborne et al. (3) by using autoradiography techniques have provided evidence that the main localization of the binding sites was the mesangial cells. The physiological significance of this binding is not completely known. Experiments have however shown that concentrations of AII similar to those at which binding occurs in vitro can produce a reduction in glomerular diameter and glomerular capillary radius (1, 4). Such an effect may be mediated by activation, perhaps *via* the AII receptor, of the contractile properties of the mesangial cells (5). Very recent evidence suggests yet other potential roles for AII on glomerular permeability. Blantz et al. (6) with micropuncture studies in the rat observed that AII produced a fall in single nephron glomerular filtration rate related to a decrease in total glomerular permeability. This decrease was considerably greater than expected on the basis of a reduction in the glomerular surface area (associated to the decrease in capillary radius) and therefore suggested a change in local capillary permeability to water. Eisenbach et al. (7) have also shown

[1] *Abbreviations used in this paper:* AII, angiotensin II; GBM, glomerular basement membrane; k_{+1}, association constant; k_{-1} dissociation constant; K_D, ratio of k_{+1} and k_{-1}.

TABLE I

Fraction of the Glomerular Receptors for Angiotensin II Present in GBM

Experimental procedure	Ratio of ^{125}I-AII binding to* GBM over that to glomeruli	Ratio of GBM protein over glomerular protein	Ratio of GBM receptors over glomerular receptors
	%	%	%
Binding as a function of ^{125}I-AII concentration	172	8.0	13.8
Binding as a function of unlabeled AII concentration	216	10.0	21.6
Idem	216	10.0	15.9
Time-course study	271	12.2	33.1
Idem	259	6.2	16.1
Mean±SEM	251±22	9.3±1.0	20.1±3.5

* This ratio was calculated with the data obtained after a 20-min incubation at 50 pM ^{125}I-AII.

that AII can increase glomerular permeability to proteins. Since glomerular basement membrane (GBM) is considered as the main barrier in the process of glomerular filtration, this prompted us to look for specific receptors for AII in GBM fractions isolated from rat renal glomeruli.

METHODS

Materials. (Asn^1, Val^5) AII was donated by Ciba-Geigy (Basel, Switzerland) and labeled with ^{125}I by the method of Hunter and Greenwood (8). Labeled and unlabeled molecules were separated by using polyacrylamide gel electrophoresis according to Corvol et al. (9). The specific activity of the labeled hormone, assessed by radioimmunoassay as previously described (1) was 2,000 mCi/μmol. This is close to the theoretical value corresponding to one atom iodine per molecule. The following chemicals were purchased from the commercial sources indicated: neuraminidase (from *Vibrio cholerae*, 500 U/ml) and trypsin (10,340 U per mg) from Calbiochem, Los Angeles, Calif.; soybean trypsin inhibitor from Sigma Chemical Co., St. Louis, Mo.; collagenase (537 U/mg) from Worthington Biochemical Corp., Freehold, N. J.; 1–34 bovine PTH, (Asp^1, Ile^5) AII, (Des Asp^1, Ile^5)AII, (Des Asp^1, Des Arg^2, Ile^5)AII, (Sar^1, Ile^8)AII from Beckman Instruments, Inc., (Cedar Grove, N. J.). Salmon calcitonin was a gift from Armour Pharmaceutical Co. (Kankakee, Ill.) and porcine monocomponent insulin from Novo Research Institute (Copenhagen, Denmark).

Preparation of isolated glomeruli, GBM, and anti-GBM antibodies. Renal glomeruli were isolated from female Sprague Dawley rats of 150–220 g body weight according to the technique of Fong and Drummond (10) with minor modifications. In short, cortex from four kidneys was dissected and minced to a paste-like consistency. The homogenate was then gently pushed through a 106 mesh screen and suspended in 10 ml of 0.07 M phosphate buffer, pH 7.5. The suspension was passed through a 25 gauge needle and then centrifuged at 120 *g* for 90 s. The supernate was discarded and the pellet resuspended in the same buffer solution, passed again through the needle, and centrifuged. This operation was repeated five times to obtain the final glomerular preparation. GBM were purified from the isolated glomeruli according to the technique of Blau and Michael (11); isolated glomeruli were sonicated with a Branson sonifer (Branson Sonic Power Co., Danbury, Conn., model W185 D) at position 5 for 3 min. The sediment of washed GBM obtained at the end of the purification process was either lyophilized and stored for further immunization of rabbits or resuspended in 1.0 M sodium chloride and immediately used. An aliquot of sonicated glomeruli was conserved for comparison with GBM in the binding studies of AII. The protein in a given amount of isolated glomeruli and in the corresponding purified GBM was determined according to Lowry et al. (12). This allowed the calculation of the yield of purification of GBM which was about 10% (Table I). DNA (13) and phospholipids (14) were measured in both preparations and related to the dry weight. Amino-acid analysis was also performed with a Technicon (Technicon Instruments Corp., Tarrytown, N. Y.) amino-acid analyzer on protein hydrolysates (6 N HCl for 24 h at 110°C) according to the method of Spackman et al. (15). 3 OH and 4 OH-proline were separately measured with a Beckman (multichrom) analyzer according to Benson and Patterson (16). The ratio of the concentrations of these different substances in GBM and isolated glomeruli, respectively, was used to appreciate GBM purification.

Anti-GBM antibodies were raised in rabbits. Approximately 10 mg (dry weight) of GBM in 0.5 ml of phosphate buffered saline: Na H_2 PO_4, 2.4 mM; Na_2 H PO_4, 7.6 mM; Na Cl, 126 mM mixed with an equal volume of complete Freund's adjuvant was injected three times at 1-wk interval into each of four adult male rabbits. These animals received simultaneously 0.2 ml of *Bordetella pertussis* vaccine (Parke Davis and Co., Detroit, Mich.).Rabbits were then repeatedly bled, and sera were tested for anti-GBM antibody by the indirect immunofluorescence technique. Kidney sections from normal rats were first incubated with progressive dilutions of rabbit antiserum, washed, and then incubated in the presence of fluorescent antirabbit IgG. The titer of the rabbit antiserum was the greatest dilution providing a positive immunofluorescence and was 1/400 for the antiserum selected. Immune rabbit gammaglobulin was partially purified from the total serum by precipitation with 50% saturated ammonium sulfate, redissolved in phosphate buffered saline, and dialyzed against repeated changes of this buffer during 24 h. Similarly isolated normal rabbit IgG served as control. The final preparations contained approximately 20 mg/ml of protein. To study the specificity of the antibody, anti-GBM IgG was further purified by DEAE cellulose (Whatman DE 52) chromatography with 0.01 M sodium phosphate buffer, pH 7.4, and labeled with horseradish peroxidase according to

Avrameas (17). The peroxidase labeled IgG was separated from free peroxidase by chromatography on Sephadex G 200 (Pharmacia Fine Chemicals, Inc. Piscataway, N. J.), concentrated, and adjusted to 5 mg/ml. Two 70-g rats unilaterally nephrectomized were injected intravenously with 1 ml of the conjugate and were sacrificed 3 days later. Renal tissue was processed for electron-microscopy study, peroxidase activity was demonstrated by conventional techniques (17).

In vitro AII binding studies. AII binding was studied with either GBM or sonicated isolated glomeruli incubated in the presence of 50 pM ^{125}I-AII at 20–22°C. The incubation milieu was 0.2 M NaCl, 0.014 M PO_4 H_2 K, 0.056 M PO_4 H Na_2, pH 7.5. Bovine serum albumin and 1–24 ACTH fragment (Ciba-Geigy) at final concentrations of 2% and 125 μg/ml were added. The latter product, as shown in a previous study (1), prevented degradation of the tracer without affecting binding kinetics. Bound radioactivity was separated with filtration through a Millipore filter (Millipore Corp., Bedford, Mass.) (HAWP 02500). ^{125}I was counted with a crystal type scintillation detector giving 30% efficiency. Specific binding was calculated by subtracting the binding in the presence of 50 μM unlabeled AII from total binding and expressed as femtomoles of bound hormone per milligram of glomerular or GBM protein. This mode of expression was valid for both preparations. It has been shown in a previous study (1) that the amount of AII bound to isolated glomeruli was linearly related to the amount of glomerular protein over the range of protein concentrations studied (100–500 μg/ml). Similar linearity was also obtained when AII bound to GBM was plotted against the amount of GBM protein ($r = +0.995$).

In other experiments, GBM were exposed first to anti-GBM antibodies, washed extensively, and then incubated with ^{125}I-AII. Precisely, 0.4 ml of GBM suspension (2.2 mg of protein/ml) was centrifuged and the corresponding pellet incubated in a Dubnoff incubator with continuous agitation during 1 h at 4°C with an equal volume of phosphate buffered saline containing partially purified IgG (20 mg/ml) obtained from either immunized or control rabbits. The preparation was then centrifuged at 120 *g* for 15 min. After the supernate was discarded, the pellet was washed twice with distilled water, resuspended in 1.0 M sodium chloride, and used in binding experiments in the same conditions as stated above.

In vivo binding studies. In each of these experiments two rats were injected intravenously with 60 mg (3 ml) of IgG partially purified from the serum of immunized rabbits and two other rats with the same amount of partially purified IgG from control rabbits. 16 h later, a blood and a urine sample were obtained for assay of creatinine and protein, respectively. Creatinine was measured by the usual technique on a Technicon autoanalyzer and protein was measured by turbidimetry. The kidneys were then removed and the renal glomeruli isolated but not sonicated. A portion of each final preparation was incubated in the presence of fluorescent antirabbit IgG to verify the binding of anti-GBM antibodies to the glomeruli from immunized rats and the absence of binding in control rats. Fig. 1*a* shows the linear deposition of anti-GBM antibodies along GBM obtained in the immunized rats. AII binding to glomeruli isolated from either passively immunized or control rats was studied as stated above except for the incubation milieu which was 0.07 M phosphate buffer, pH 7.5.

Spontaneous degradation of ^{125}I-AII. Degradation of ^{125}I-AII present in the incubation milieu was studied with polyacrylamide gel electrophoresis and binding to an excess of specific antibodies. Polyacrylamide gel electrophoresis was performed according to Corvol et al. (9). Undamaged hormone was estimated as the peak of radioactivity corresponding to ^{125}I-AII Rf. Binding of ^{125}I-AII to an excess of specific antibody was measured after a 16-h incubation. This antibody had been raised in the rabbit and was used at a final dilution of 1/400 which produced binding of more than 90% of the total radioactivity. Free and bound radioactivities were separated with the charcoal dextran method. In each experiment, appropriate controls were prepared which were identical except that isolated glomeruli or GBM were omitted. These controls represented 100% of the hormone available for degradation. To evaluate hormone degradation, results were expressed as percentage of control remaining after the same period of incubation.

Nature of the receptor. GBM (1 mg of protein/ml) were incubated with various enzymes during 30 min in 1.0 M NaCl. Final concentrations of the enzymes were 200 μg/ml for collagenase, 2,500 μg/ml for trypsin, and 12.5 IU/ml for neuraminidase. Temperature was 37°C for the first and 30°C for the latter two. After incubation the tubes were centrifuged (5 min at 500 *g*) and the pellet washed once with 1.0 M NaCl. Treated and control membranes (100 μg of protein) were then added to tubes containing 100 pM ^{125}I-AII and incubated during 20 min in the same conditions as stated above. In the trypsin studies. parallel experiments were performed: soybean trypsin inhibitor (2,500 μg/ml) was added to either treated or control membranes to inhibit any trace of trypsin left after washing which could degrade the hormone.

RESULTS

Tests of validity of purification of GBM. The preparation of GBM was considered as purified from the following tests. Firstly, electron microscopy examination (Fig. 2) showed that GBM were free of cells or recognizable fragments but small amounts of cellular debris were seen, probably trapped between the isolated membranes during centrifugation. Since the preparation of isolated glomeruli was free of Bowman's capsule, GBM can be considered as only coming from the capillary walls. Secondly, chemical markers were assayed in both preparations (Table II) and the results obtained were close or similar to published biochemical data on GBM (18, 19). The concentration of DNA considered as a nucleus marker was 103 ±15 μg/mg of dry weight in the starting preparation of isolated glomeruli and equal to or below the lower limit of detection in the GBM preparation. Similarly, the concentration of phospholipids which are a cell membrane marker was 52.0±3.4 μg/mg of dry weight in the glomerular preparation and 11.2±1.2 μg/mg of dry weight in the GBM preparation. The latter value is close to that found by Sato et al. (18) and smaller than that observed by Westberg and Michael (19) in human GBM. The ratio of purification of GBM was in the same range as in the latter study. Amino-acid analysis showed that GBM contained higher concentrations of hydroxyproline, hydroxylysine, and glycine than isolated glomeruli

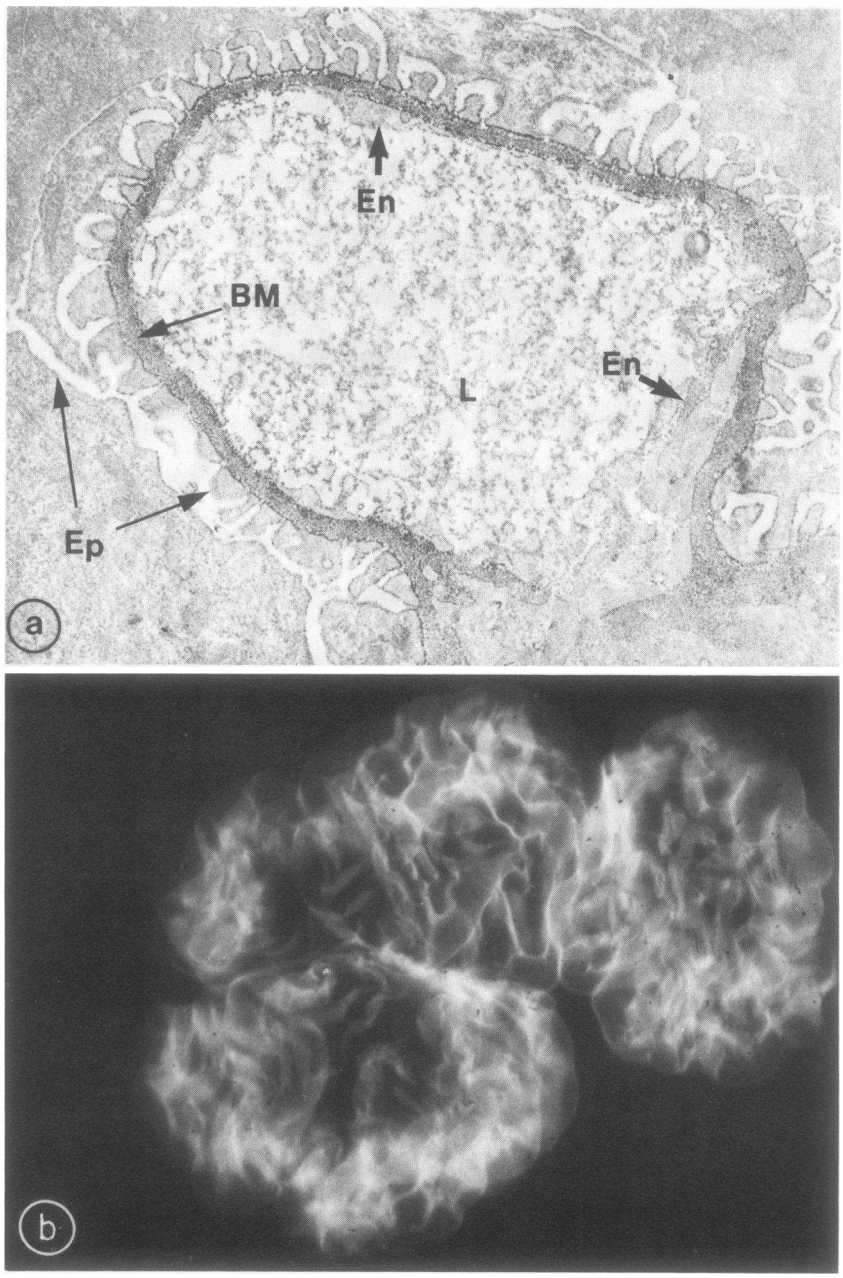

FIGURE 1*a* and 1*b* Linear IgG is seen along GBM of isolated glomeruli from a rat immunized with anti-GBM antibodies raised in the rabbit. Fluorescein isothiocyanate conjugated anti-rabbit IgG was used. Original magnification, ×400 (*a*). Electron micrograph of a kidney section from a rat sacrificed 3 days after injection of peroxidase-conjugated antibodies to GBM. L, capillary lumen; En, endothelial cell; Ep, epithelial cell; BM, basement membrane. Note intense labeling of the basement membrane while endothelial and epithelial cell membranes remain unlabeled. Original magnification, ×18,900 (*b*).

(18, 19). The enrichment factors were in agreement with the data of the literature. The basement membrane specific 3 hydroxyproline represented more than 20% of total hydroxyproline as found by Westberg and Michael (19). Thirdly, GBM were incubated with either anti-GBM antibodies or IgG fraction from control rabbits, washed, and then exposed to fluorescent antirabbit IgG in the same conditions as above

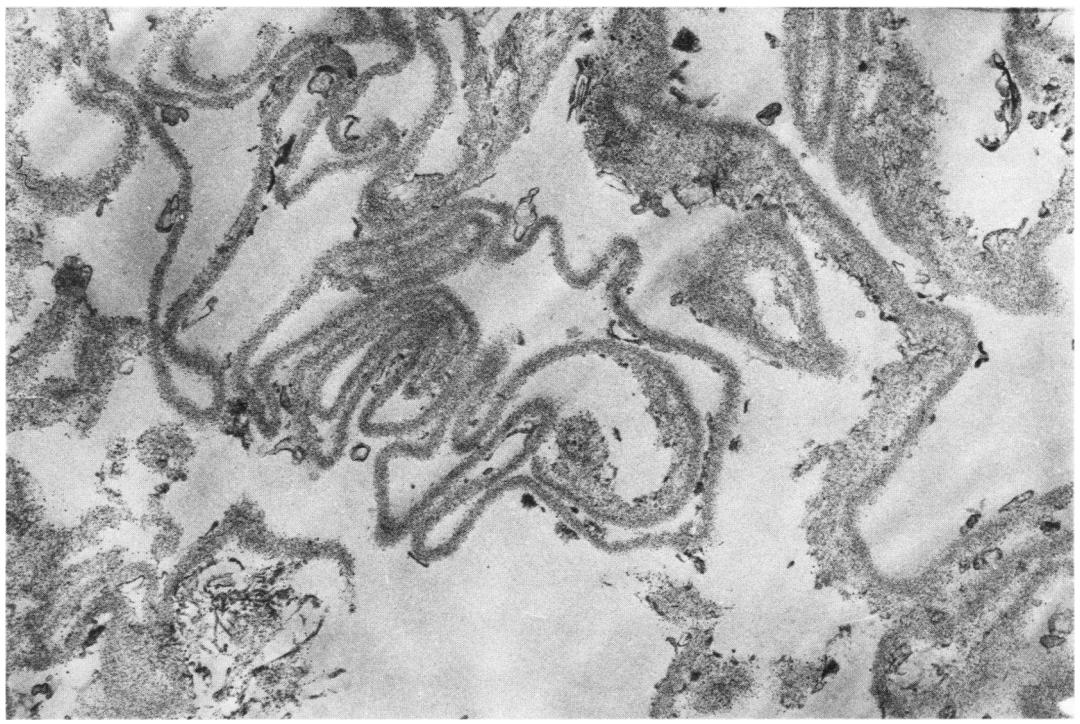

FIGURE 2 Electron micrograph of the pellet of centrifugation of GBM. Some cellular remnants and scarce collagen fibers are seen between numerous basement membranes. Original magnification, ×7,300.

for isolated glomeruli. Fluorescence was obtained only with the preparation incubated with anti-GBM antibodies.

In vitro studies of ^{125}I-AII binding to GBM and isolated glomeruli. ^{125}I-AII binding was measured as a function of time at a concentration in the milieu of 50 pM in the presence of either isolated glomeruli or GBM in parallel experiments (Fig. 3). Steady state was obtained after 16 min with both preparations. Specific binding related to the amount of receptor

TABLE II
Purification of GBM

	Isolated glomeruli	GBM	Ratio GBM over isolated glomeruli
DNA, *μg/mg of dry weight*	103 (3)	Below the detection limit	
Phospholipids, *μg/mg of dry weight*	50.2 (3)	11.2 (3)	0.215
Total hydroxyproline, *nmol/mg of dry weight*	52.5 (2)	136.2 (2)	2.59
$\frac{\text{3-Hydroxyproline}}{\text{Total hydroxyproline}}$	3-Hydroxyproline below the detection limit	0.25 (2)	
Hydroxylysine, *nmol/mg of dry weight*	14.2 (2)	63.6 (2)	4.47
Glycine, *nmol/mg of dry weight*	170.3 (2)	349.3 (2)	2.07

Values given are means. Numbers of determinations are indicated between brackets.

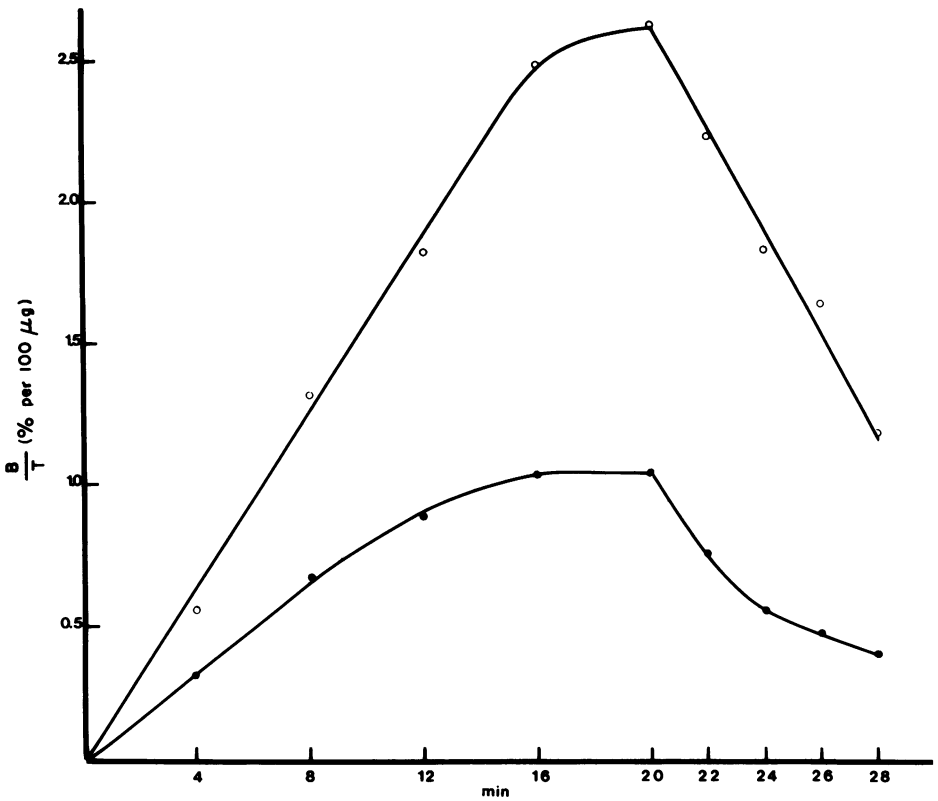

FIGURE 3 Binding of ^{125}I-angiotensin II (50 pM) to glomerular basement membrane (open circles) and to sonicated isolated glomeruli (closed circles) as a function of time. Each point is the mean of duplicates. After a 20-min incubation, unlabeled angiotensin II (50 μM) was added to study the dissociation of bound hormone from its receptors. Bound hormone is expressed as B/T per 100 μg of protein. B/T is the ratio bound over total radioactivity. Total radioactivity present in the incubation milieu was multiplied at each time by the corresponding fraction of intact hormone measured in parallel experiments.

protein was clearly higher in the preparation of purified GBM. Dissociation of the bound hormone was produced in both preparations by the addition of 50 μM unlabeled AII, and at any time, the residual bound hormone was greater with the preparation of GBM. This higher binding of AII to GBM was not due to a greater degradation of ^{125}I-AII in the incubation milieu since bound hormone was expressed as the percentage of the concentration of undegraded hormone in the milieu. This concentration at each time was obtained by multiplying the total radioactivity concentration by the corresponding fraction of intact hormone measured in parallel experiments. The latter mode of expression allowed the correction of the data according to the different rates of degradation of ^{125}I-AII in the presence of GBM or isolated glomeruli (see below: degradation studies). ^{125}I-AII binding was also measured as a function of hormonal concentration after a 20-min incubation. An apparent nonlinear curve was obtained both with GBM and isolated glomeruli preparations for concentrations of ^{125}I-AII ranging from 50 pM to 4 nM (Fig. 4*a*). It could reflect a cooperative model in which the affinity of binding is modified with the degree of occupancy of the receptor sites. Binding was clearly higher for all points of the curve with the GBM preparation. Inhibition of binding of ^{125}I-AII by unlabeled AII was compared in both preparations. As shown in Fig. 5*a*, dilution of ^{125}I-AII (50 pM) with increasing concentrations of unlabeled AII above 0.01 ng/ml (10 pM) decreased the percentage bound of the total hormone in both preparations. As indicated for the time-course studies, total hormone, at each concentration of unlabeled hormone, was corrected for degradation measured in parallel experiments. At concentrations of native AII ranging between 0 and 1 μg/ml, binding was clearly higher in the GBM preparation, but the difference between the two curves diminished progressively when the concentration of unlabeled AII increased. The indexes of binding were calculated from time-course studies as previously described (1). Table III shows the values of the association constant (k_{+1}),

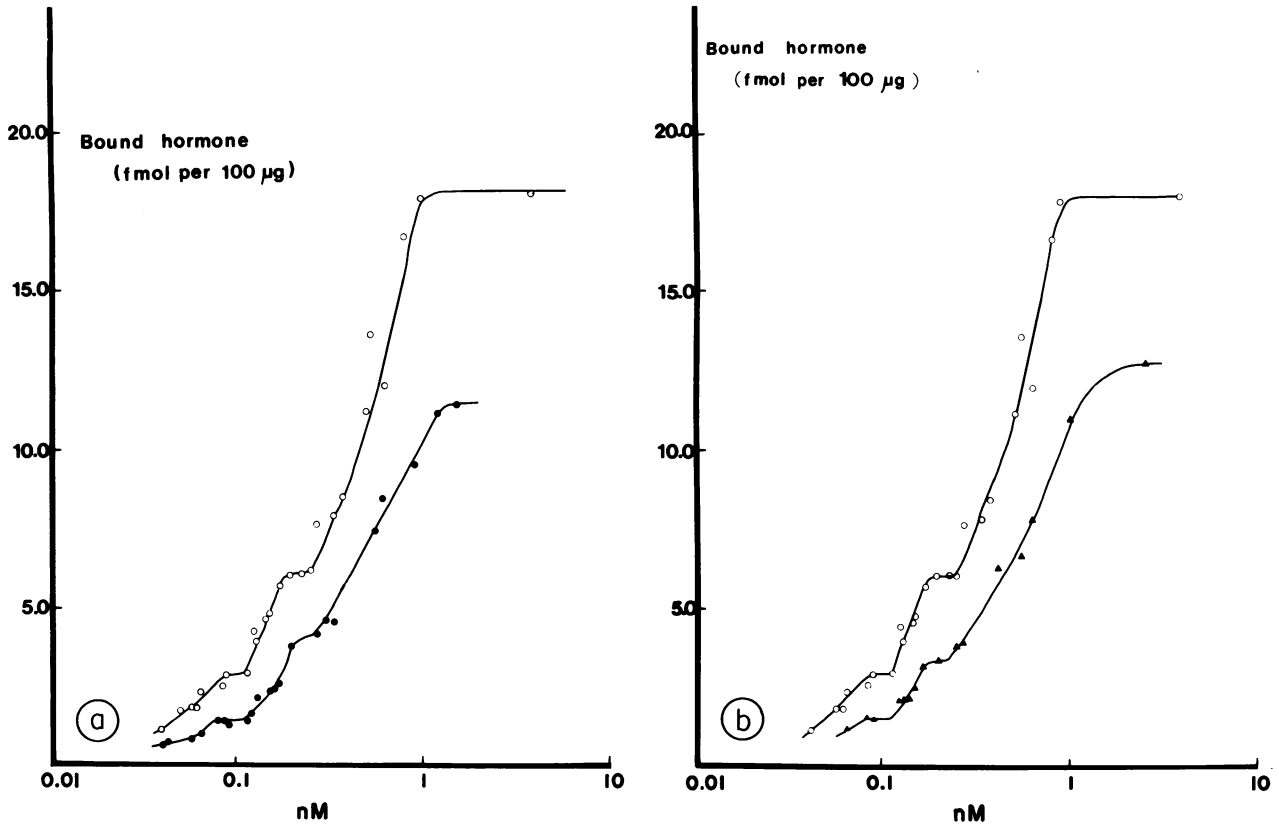

FIGURE 4*a* and 4*b* Binding of ^{125}I-angiotensin II as a function of the concentration of labeled hormone in the incubation milieu. On the right (*b*), open circles represent binding of GBM and closed triangles binding to sonicated isolated glomeruli. On the left (*a*), closed and open circles represent binding to GBM after preincubation with IgG partially purified from anti-GBM serum and control serum, respectively. Each point is the mean of duplicates.

the dissociation constant (k_{-1}), and the ratio (K_D) of these two parameters. There was no difference between the indexes of binding obtained with GBM and isolated glomeruli, respectively. K_D was close to 50 pM with both preparations. K_D could also be roughly estimated from binding studies at equilibrium as the concentration corresponding to 50% of the estimated value of the apparent plateaus. Considering only the higher affinity group of receptor sites, K_D derived from the data presented in Fig. 4*b* are approximately 50 pM for both preparations thus confirming the values obtained from kinetic studies. Number of sites and K_D were finally calculated using the Scatchard's transformation of the data obtained from competitive binding experiments. The experimental curves could be resolved into two straight lines corresponding to two groups of binding sites with the GBM as well as with the isolated glomeruli preparations (Fig. 5*b*). The two straight lines with the highest slopes were parallel demonstrating similarity of K_D for both preparations. These K_D were approximately one order of magnitude greater than those calculated from time-course studies or from binding studies at equilibrium as observed previously (1). The number of sites corresponding to the highest affinity groups was calculated from the abscissa intercept and found clearly greater with the GBM preparation (Table III). The effect of the concentration of the binding sites on AII binding was also investigated. Binding was linearly related to the amount of glomerular ($r = +0.970$) or GBM protein ($r = +0.995$) in the range of the concentrations tested. The slope of the regression line corresponding to GBM (0.027 fmol/µg) was twice that obtained with isolated glomeruli (0.013 fmol/µg). The specificity of AII binding sites was indicated by the lack of binding-inhibition activity of unrelated peptides including 1–34 bovine parathyroid hormone, salmon calcitonin, porcine insulin, in contrast with the binding-inhibition potency of (Asn^1, Val^5)AII, and (Asp^1, Ile^5)AII. These two forms of AII were approximately equipotent. The 2–8 heptapeptide (Des

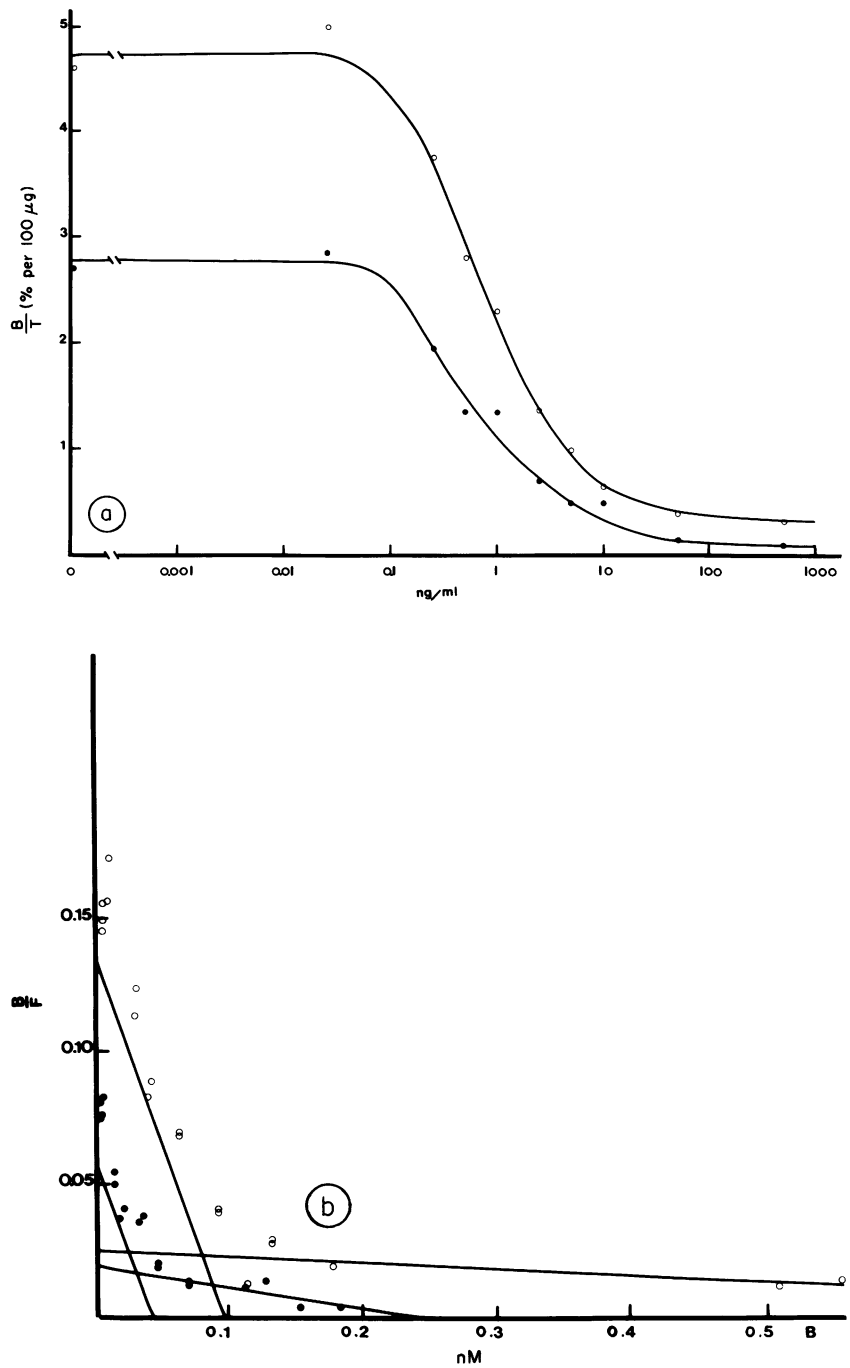

FIGURE 5 Inhibition of binding of ^{125}I-angiotensin II (50 pM) to GBM (open circles) and to sonicated isolated glomeruli (closed circles) as a function of the concentration of unlabeled angiotensin II (*a*). Each point is the mean of duplicates. The same data is expressed with the Scatchard's plot, i.e. the ratio bound over free hormone versus bound hormone (*b*). Each of the experimental curves was decomposed into two straight lines as follows: the regression line corresponding to the six greater concentrations of bound hormone was calculated and subtracted from the other experimental points to obtain the greater slope line. The two greater slope lines corresponding to GBM and isolated glomeruli, respectively, were considered as reflecting high affinity groups of sites. The linearity of these two curves was confirmed by the values of the correlation coefficients (−0.940 and −0.920 for GBM and isolated glomeruli, respectively) which were close to unity.

TABLE III

Indexes of Binding of ^{125}I-AII to GBM and to Sonicated Isolated Glomeruli (G) Calculated from Paired Experiments

Experimental procedure	k_{+1}		k_{-1}		K_D		Number of sites	
	GBM	G	GBM	G	GBM	G	GBM	G
	$M^{-1}\ min^{-1}$		min^{-1}		nM		*fmol/mg*	
Time-coure study	0.46×10^{10}	0.71×10^{10}	0.114	0.240	0.02	0.03	—	—
Idem	0.11×10^{10}	0.23×10^{10}	0.083	0.104	0.08	0.05	—	—
Idem	0.21×10^{10}	0.31×10^{10}	0.082	0.125	0.04	0.04	—	—
Competitive binding experiment	—	—	—	—	0.73	0.70	330	153
Idem	—	—	—	—	0.12	0.56	232	146

Asp^1)AII was slightly less active whereas the activity of the 3–8 hexapeptide (Des Asp^1, Des Arg^2)AII was less than half that of the intact octapeptide at the greatest concentration tested. The competitive binding inhibitor (Sar^1, Ala^8)AII displayed the same binding-inhibition potency as that of AII (Table IV).

In vitro studies of ^{125}I-AII binding to GBM exposed to anti-GBM antibodies. Binding of anti-GBM antibodies to GBM preparation after a 30-min incubation and extensive washing was always detectable by immunofluorescence whereas there was no binding with IgG fraction obtained from normal rabbits. Binding of ^{125}I-AII to GBM as a function of ^{125}I-AII concentration in the milieu was clearly diminished when GBM had been previously exposed to anti-GBM antibodies (Fig. 4*b*). The decrease was about 50% at the lower concentrations tested and was observed whatever the concentration of hormone. Apparent K_D values estimated as the concentrations corresponding to 50% of the apparent plateaus were similar with both preparations. On the contrary the number of sites calculated roughly from the amount of AII bound at each of these plateaus was clearly diminished after incubation with anti-GBM antibodies.

In vivo binding studies. Proteinuria and plasma creatinine were measured both in the experimental and in the control groups just before removing the kidneys. There was no difference in plasma creatinine (5.6±0.33 and 5.2±0.47 mg/liter in these two groups, respectively) whereas proteinuria was marked in the immunized rats (7.59±1.00 g/liter) and slight in the controls (0.33±0.07 g/liter). Immunofluorescence studies showed the linear deposition of anti-GBM antibodies along GBM in kidney sections as well as in the final preparation of isolated glomeruli (Fig. 1*a*). Electron micrographs of kidney sections (Fig. 1*b*) from animals injected with peroxidase-conjugated antibodies confirmed the presence of intense peroxidase activity throughout the GBM. Labeling was not detected on the endothelial or epithelial cells.

Binding of ^{125}I-AII to the glomeruli isolated from immunized rats was markedly decreased. In time-course studies, the plateau was much lower reaching approximately 30% of that observed with the control preparation. The two dissociation curves were parallel (Fig. 6). Binding was also measured as a function of ^{125}I-AII concentration in the milieu. At any concentration binding was clearly lower with the glomeruli isolated from immunized rats (Fig. 7). Indexes of binding were calculated from the time-course studies as stated above. The affinity of the binding sites for AII was one order of magnitude less with the preparation of immunized rats than with that of control rats ($K_D = 2.21 \pm 0.27 \times 10^{-10}$ M and $4.15 \pm 0.57 \times 10^{-11}$ m in these two preparations, respectively).

Spontaneous degradation of ^{125}I-AII. Degradation of ^{125}I-AII in the presence of either isolated glomeruli or GBM was measured with polyacrylamide gel electrophoresis and binding to an excess of specific

TABLE IV

Inhibition of ^{125}I-AII Binding to GBM by Related and Unrelated Peptides Expressed as Percentage of the Amount of ^{125}I-AII Bound in the Absence of any peptide (100±5.2)

Peptide tested	Concentration			
	0.1	1.0	10.0	100.0
	ng/ml			
(Asn^1, Val^5) AII	8.9	13.6	83.6	97.1
(Asp^1, Ile^5) AII	2.4	18.5	88.2	98.8
(Des Asp^1) AII	0	12.9	73.6	94.6
(Des Asp^1, Des Arg^2) AII	—	0.9	—	41.2
(Sar^1, Ile^8) AII	10.7	16.2	84.5	97.5
1-34 bPTH*	—	1.9	0.8	4.3
sCT*	—	3.8	0	0
Insulin	—	4.9	—	2.2

Each value is the mean of duplicates.

* bPTH, bovine parathyroid hormone; sCT, salmon calcitonin.

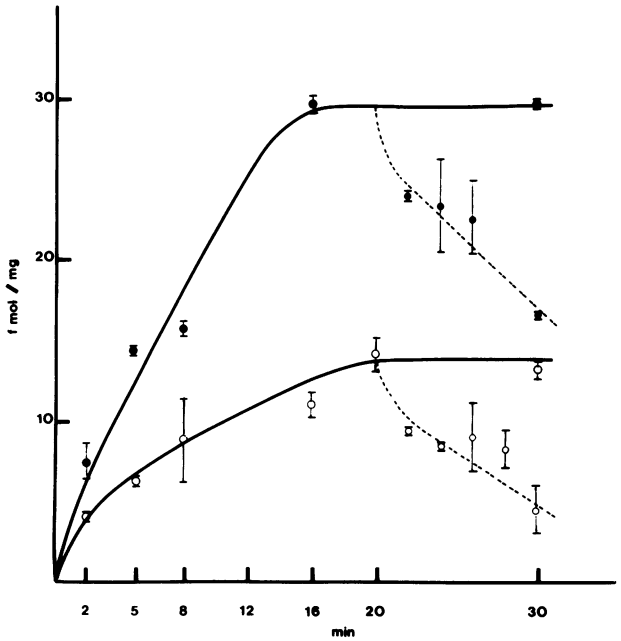

FIGURE 6 Time course of ^{125}I-angiotensin II binding to glomeruli isolated from control rats (closed circles) or rats passively immunized with anti-GBM antibodies (open circles). Each point is the mean of triplicates and the corresponding vertical bar twice the standard deviation. After a 20-min incubation, unlabeled angiotensin II (50 μM) was added to study the dissociation of bound hormone from its receptors (dotted lines).

antibodies. There was a progressive inactivation of the tracer as a function of time in the presence of isolated glomeruli whereas there was no change in the fraction of intact hormone between 5 and 15 min in the presence of GBM. Table V shows that after a 5-min incubation, there was more intact hormone in the preparation of isolated glomeruli whereas after a 15-min incubation there was more intact hormone in the preparation of GBM. The difference at any time was small, always less than 15%. This small difference could not account for the increase in binding observed with GBM. Similarly there was no difference between the rates of degradation of ^{125}I-AII in the presence of glomeruli isolated from either control or immunized rats. In both cases, the amount of degraded hormone estimated as the percentage bound to an excess of specific antibody did not exceed 20% after a 15-min incubation.

Nature of the binding sites. To determine the nature of the binding sites, GBM preparations were incubated with several enzymes and subsequently tested for their ability to bind ^{125}I-AII. Four experiments were performed with each of the three enzymes used and the results given as mean±SEM of ^{125}I-AII bound to the treated membranes. This binding was expressed as a percentage of the control binding to untreated membranes measured in parallel experiments. Treatment with collagenase produced an increase of binding, 281.5±29.0% whereas there was a slight decrease after treatment with neuraminidase, 78.0±9.0%. Addition of soybean trypsin inhibitor after washing of the preincubated membranes to inhibit any trypsin contaminant did not change the results, 5.0±0.4% showing thus that the decrease in binding was not the consequence of a possible degradation of the tracer. This interpretation was confirmed by the smaller percentage of ^{125}I-AII degraded at the end of the incubation with trypsin-treated (12.8±0.5%)

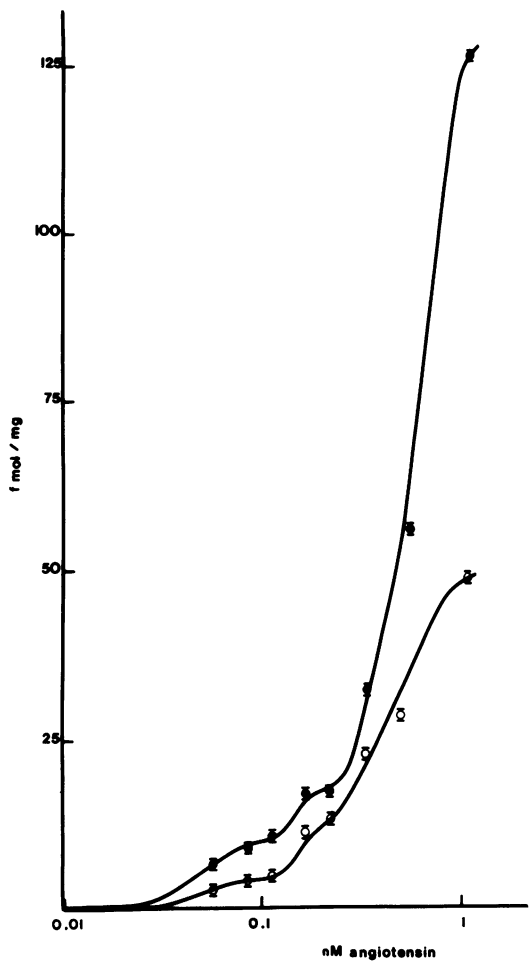

FIGURE 7 Binding of ^{125}I-angiotensin II to isolated glomeruli as a function of the concentration of labeled hormone in the incubation milieu. Glomeruli were isolated from control rats (closed circles) or rats passively immunized with anti-GBM antibodies (open circles). Each point is the mean of triplicates and the corresponding vertical bar twice the standard deviation.

than with control (21.2±1.3%) membranes, without any effect in both cases of the trypsin inhibitor.

DISCUSSION

Previous demonstration that AII binds specifically to isolated rat renal glomeruli (1, 2) left unclear the precise site of binding within the glomerulus. This study demonstrates that part of these binding sites are located in GBM, although GBM does not represent the totality of the glomerular binding sites for AII. As previously demonstrated in a study on the interaction of AII and isolated glomeruli (1), the specificity of binding to GBM relies on the following: very low calculated K_D, about 50 pM, indicating high affinity of the hormone for its receptor, reversibility of the steady-state binding after addition of unlabeled hormone, specific inhibition of binding of labeled AII by unlabeled hormone, analogues, and antagonists in contrast with the lack of inhibition in the presence of unrelated peptides. Binding activity is relatively unaffected by change in the NH_2-terminal aspartyl residue including deletion or amidation whereas it is clearly diminished by the deletion of the two first N-terminal amino acids. The indexes of binding calculated from kinetic studies are very close to those found with isolated glomeruli since k_{+1}, the k_{-1}, and their ratio K_D which measures the affinity of AII for its receptors were in the same range with both preparations. Similar K_D were derived from binding studies at equilibrium, but those calculated from competitive binding experiments with Scatchard's transformation of the data were one order of magnitude greater. As demonstrated by Jacobs et al. (20), K_D value calculated in the latter condition is overestimated when the concentrations of labeled ligand and receptor are not substantially less than the true K_D. These conditions prevailed in our competitive binding experiments since the adequate theoretical concentrations of ligand and receptor (5pM or less) would have not been compatible with a good precision of radioactive counting. When binding was related to the amount of glomerular or GBM protein, it was markedly higher with the GBM preparation. Table I shows that binding at equilibrium for 50 pM of ^{125}I-AII in the incubation milieu was about two times greater with GBM than with isolated glomeruli. This factor is in good agreement with the ratio of the number of binding sites calculated from Scatchard's plot and with the ratio of the slope of the regression lines obtained when binding was plotted against the amount of binding protein. These facts demonstrate clearly that the number of binding sites per milligram of protein was two times greater in GBM than in the whole glomerulus. The amount of GBM protein extracted from a given amount of glomerular protein was about 10% (Table I). Combining these two values, it is possible to estimate roughly the share of the GBM binding sites for AII as representing 20% of the total number of binding sites found in the entire glomerulus. The binding sites for AII present in GBM probably belong to several groups as suggested by the shape of the curve obtained when the amount of ^{125}I-AII bound was plotted against the concentration of the tracer in the incubation milieu. Moreover the Scatchard's transformation of the data allowed resolution of the experimental points into two straight lines. The line with the greatest slope and the smallest intercept corresponded to the higher affinity-lower capacity group of receptor sites which has been exclusively studied in the present work since the concentration of ^{125}I-AII chosen, 50 pM corresponded to the K_D of this group. Alternatively, the multi-plateau shape of the curve could reflect a cooperative model of the binding reaction.

TABLE V

Percentage of Degraded Hormone as a Function of Time in the Presence of Either Isolated Glomeruli (G) or GBM

Incubation time		Binding to specific antibody	Polyacrylamide gel electrophoresis
min		%	%
5	G	5.2	4.9
	GBM	10.5	19.3
10	G	11.8	15.9
	GBM	12.0	20.7
15	G	15.4	34.1
	GBM	13.4	21.5

Each value is the mean of duplicates.

Anti-GBM antibodies either injected "in vivo" or introduced "in vitro" in the incubation milieu altered the AII binding sites and impaired subsequent binding of AII. There was a 50% decrease in the amount of AII bound after incubation of GBM with anti-GBM antibodies in vitro. These antibodies might affect the hormone-binding site interaction by several mechanisms. The antibody might occupy the binding site directly or bind to the GBM on or near the AII binding site producing steric hindrance of AII binding. The antibody might also interact with a membrane component distant from the binding site, inducing a change in the membrane which subsequently alters the AII-binding site interaction. These two possibilities might intervene simultaneously. In the "in vivo" experiments, the decrease in binding of AII was greater than in the "in vitro" studies, approxi-

mately 70% and there was also a lower affinity of AII for its binding sites whereas in the "in vitro" experiments affinity was unchanged. The binding of anti-GBM antibodies to GBM was probably the initial step of an inflammatory process which accounts for the supplementary decrease in the number of AII binding sites and for the change of affinity. There are some examples in the literature of the interaction of antibody with specific hormone receptors located on the cell surface (21, 22). In the present study, the antibodies used were directed against unknown antigens of GBM and not against the specific receptors for AII themselves. In spite of this, rats passively immunized with anti-GBM antibodies can be considered as a model of "receptor disease" and used to determine the physiological significance of these receptors.

To our knowledge, the demonstration of high affinity specific binding sites for AII in GBM is the first example of localization of such binding sites for a polypeptide hormone in a noncellular structure since usually these sites are only present on the cell surface. It could theoretically be possible that AII did not bind to a constituent of GBM itself but to a plasma contaminant trapped in GBM since it has been previously shown that preparations of GBM might contain in significant amounts some plasma glycoproteins (19). This hypothesis seems unlikely because the affinity of most hormones to plasma protein is much lower than their affinity to specific cell receptors (23). AII has been shown to bind to human plasma protein but only in 5% of the subjects tested (24). Although complete chemical characterization of the binding sites is not available, some information can be drawn from the influence on binding of various enzymes. The loss of ^{125}I-AII binding after the treatment of GBM with trypsin suggested that this binding site was a protein. The marked increase in binding produced by collagenase indicated that the binding sites were not localized in the collagenous fraction of the GBM but rather in the glycoprotein moiety and were made more accessible after collagenase digestion. The slight decrease in binding produced by neuraminidase further suggested that sialic acid may be a part, probably not essential, of the binding protein.

In summary, the present studies have shown that AII can bind specifically and with a high affinity to the glycoprotein moiety of the GBM. We have no evidence to demonstrate that the binding sites involved in this reaction are real receptors mediating a subsequent physiological event. However, since GBM is the main barrier to glomerular filtration, it can be suggested that interaction of AII with GBM may be the first event in a change in glomerular permeability induced by AII postulated in recent works with micropuncture data (6, 7) or clearance studies (25).

ACKNOWLEDGMENTS

We thank Drs. Callard, Druet, and Laliberté for performing the electron-microscopy examinations, Dr. Morel-Maroger for her help in immunofluorescence, Dr. Alcindor for phospholipid assay, and Dr. Muh for amino-acid analysis. The authors thank Miss M. Bizien for secretarial assistance.

This work was supported by Institut National de la Santé et de la Recherche Médicale, and by a grant (no. 75 7 1289) of the Délégation Générale à la Recherche Scientifique et Technique.

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