EVIDENCE FOR MESANGIAL GLOMERULAR RECEPTORS FOR ANGIOTENSIN II LINKED TO MESANGIAL CELL CONTRACTILITY

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1. Introduction

Specific receptors for angiotensin II (A II) have been shown in glomeruli isolated from rat kidney cortex and related to glomerular vaso-reactivity [1]. The localization of A II receptors within the glomerulus and the relationship between these receptors and the physiological functions of the corresponding cells are still unknown. After injection of tritiated A II into the renal artery of the rat, ^3H radioactivity was observed by autoradiography in the mesangial cells [2], but the physiological significance of this uptake remained imprecise. High affinity binding sites for A II on rat glomerular basement membranes were observed [3] but no experimental data allowing correlation of this binding with a physiological function was provided. Homogeneous populations of glomerular epithelial and mesangial cells have now been cultured [4,5]. It is thus possible to study the biochemical properties of these two different glomerular cell populations. We have shown that the mesangial cells represent the main site of prostaglandin (PG) E_2 glomerular synthesis [6]. Here, we provide evidence for localization of specific A II receptors on the mesangial cells and demonstrate that the binding of A II to these receptors induces a contractile response of mesangial cells.

2. Experimental

2.1. *Materials*

(Asn^1, Val^5) A II was donated by Ciba-Geigy (Basel) and labeled with ^{125}I by the method in [7]. Labeled and unlabeled molecules were separated by using polyacrylamide gel electrophoresis according to [8]. The specific activity of the labeled hormone assessed by radioimmunoassay was 2000 Ci/mmol. This is close to the theoretical value corresponding to 1 iodine atom/molecule. The following peptides were purchased from the commercial sources as indicated: (Sar^1, Ala^8) A II, (Sar^1, Ile^8) A II, (Des Asp^1, Ile^5) A II and 1–34 bovine PTH from Beckman (Geneva); lysine-vasopressin from Sandoz (Basel); 1–24 ACTH fragment from Ciba-Geigy. One synthetic analogue of A II (Des Asn^1, Val^5) A II was kindly provided by Professor Riniker (Ciba-Geigy).

2.2. *Cell cultures*

Renal glomeruli were isolated from Sprague-Dawley rats (150–220 g) as in [4]. The final preparation contained purified glomeruli without Bowman's capsules and afferent or efferent arteries. These glomeruli were cultured at 37°C in plastic flasks containing 5 ml RPMI medium (Flow Labs., Irvine), supplemented with 10% decomplemented fetal bovine serum, and buffered with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes, pH 7.2). Epithelial cells grow rapidly with a peak of cell division on day 6 whereas mesangial cells grow at a slower rate with a maximum of cell division on day 22. These 2 cell populations were therefore separated by subculture at these 2 different times. The conditions of the subcultures were identical to those used for isolated glomeruli. Culture media were changed every 2 days. For the binding experiments, the epithelial and the mesangial cells were tested 5 and 15 days, respectively, after initiation of the subculture. These dates correspond to the maximum recovery by these cells of their biological properties [4,6,9].

2.3. *Binding experiments*

Binding experiments were done using dissociated cells scraped off their flasks. This technique allows

precise replication of the binding studies, since uniform aliquots of the starting cell suspension are used.

Flasks containing mesangial or epithelial cells were washed with 5 ml of Dulbecco's solution (137 mM NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 5.6 mM glucose, pH 7.2) and then exposed to 5 ml of this same solution containing 0.5 mM EDTA (disodium salt) for 3 min at 22°C. This medium was removed and replaced by 5 ml Dulbecco's solution containing 380 IU/ml type I collagenase and 1 mg/ml soybean trypsin inhibitor (Sigma, St Louis MO). Incubation with the latter medium was performed at 37°C for 10 min. The supernatant was aspirated and kept. Hank's balanced salt solution (pH 7.2) (no. 19-101-54, Flow Labs.) 5 ml, containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$ (buffer A) were then added to the flask and the cells were scraped away using a rubber policeman. The suspension obtained and the supernatant collected after collagenase treatment were filtered through a 50 μm sieve. The filtrate was then centrifuged at 120 × *g* for 10 min and the supernatant discarded. The pellet was washed once again in the same conditions after addition of 5 ml buffer A. Binding studies were then done under 100 μl buffer A supplemented with 2 g/100 ml bovine serum albumin (Sigma) pre-heated at 56°C for 30 min and 125 μg/ml of 1–24 ACTH fragment. As shown in [1], the latter product inhibits degradation of the tracer without affecting binding kinetics. ^{125}I-labeled A II (^{125}I-A II) was added at 0.5 nM and incubated with 20–40 μg cellular protein/tube at 20–22°C for 40 min. At the end of the incubation, bound radioactivity was separated using filtration through a Millipore filter (0.22 μm GS). ^{125}I was counted with a crystal type scintillation detector giving 63% efficiency. An aliquot of the cell suspension was also used for protein determination [10]. Specific binding was calculated by subtracting the binding in the presence of 5 μM unlabeled A II from total binding and expressed as fmol bound hormone/mg cellular protein. To verify that the drugs used to dissociate the cells (disodium EDTA and collagenase) did not degrade the receptors for A II, isolated glomeruli were treated similarly to the cells. Binding studies were performed in parallel using control and treated isolated glomeruli. No inhibition of binding was observed but, on the contrary, a slightly greater amount of ^{125}I-A II bound to the treated glomeruli.

2.4. *Degradation studies*

Degradation of ^{125}I-A II present in the incubation medium was studied using either the binding to an excess of specific antibodies or the rebinding to freshly isolated glomeruli [1]. Binding of ^{125}I-A II to an excess of specific antibody was measured after 16 h incubation. This antibody had been raised in a rabbit and was used at a final dilution of 1/400, at which >90% of the total radioactivity was bound. It could not distinguish between A II and (Des Asp^1, Ile^5) A II but only slightly crossreacted with the smaller fragments of the hormone. Free and bound radioactivities were separated by using Dextran-coated charcoal. Rebinding of ^{125}I-A II to freshly isolated glomeruli was measured after 40 min incubation in the same conditions as those used for the cells. In each experiment, appropriate controls were prepared which were identical except that cells were omitted. These controls represented 100% of the hormone available for degradation. To evaluate hormone degradation, results were expressed as % of control activity remaining after the same incubation period.

2.5. *Evaluation of cellular reactivity*

Reactivity of cultured epithelial or mesangial cells to A II was first studied by phase contrast microscopy as in [9]. In short, the cultured cells were first incubated at 37°C for 1 h with 5 ml buffer A. A II was then added at 10 pM–50 nM. The changes in length, shape or phase lucency were observed within 15 min after introduction of the agonist. Cultured cells incubated in the presence of buffer A without subsequent addition of A II were used as controls.

The myosin distribution in contracting mesangial cells is quite different from that observed in non-contracting mesangial cells, as shown by indirect immunofluorescence using a specific antimyosin antiserum [4]. Indeed no prominent fibrillar structures could be detected in the cytoplasm of the contracting mesangial cells (fig.1). Therefore, the cellular reactivity was also studied by indirect immunofluorescence. Mesangial cells were grown to confluency on glass cover slips in the same culture medium as that used in the plastic flasks. Cover slips with cell monolayer were washed 3 times with buffer A (15 min each time). The cells were then incubated for 15 min at 37°C with this same buffer containing A II at 10 pM–50 nM. At the end of the incubation, cell preparations were fixed for 5 min in absolute methanol at 4°C and washed 3 times with buffer A (5 min each time). The cells were then exposed for 30 min to an antiserum directed against myosin and stained using an indirect immuno-

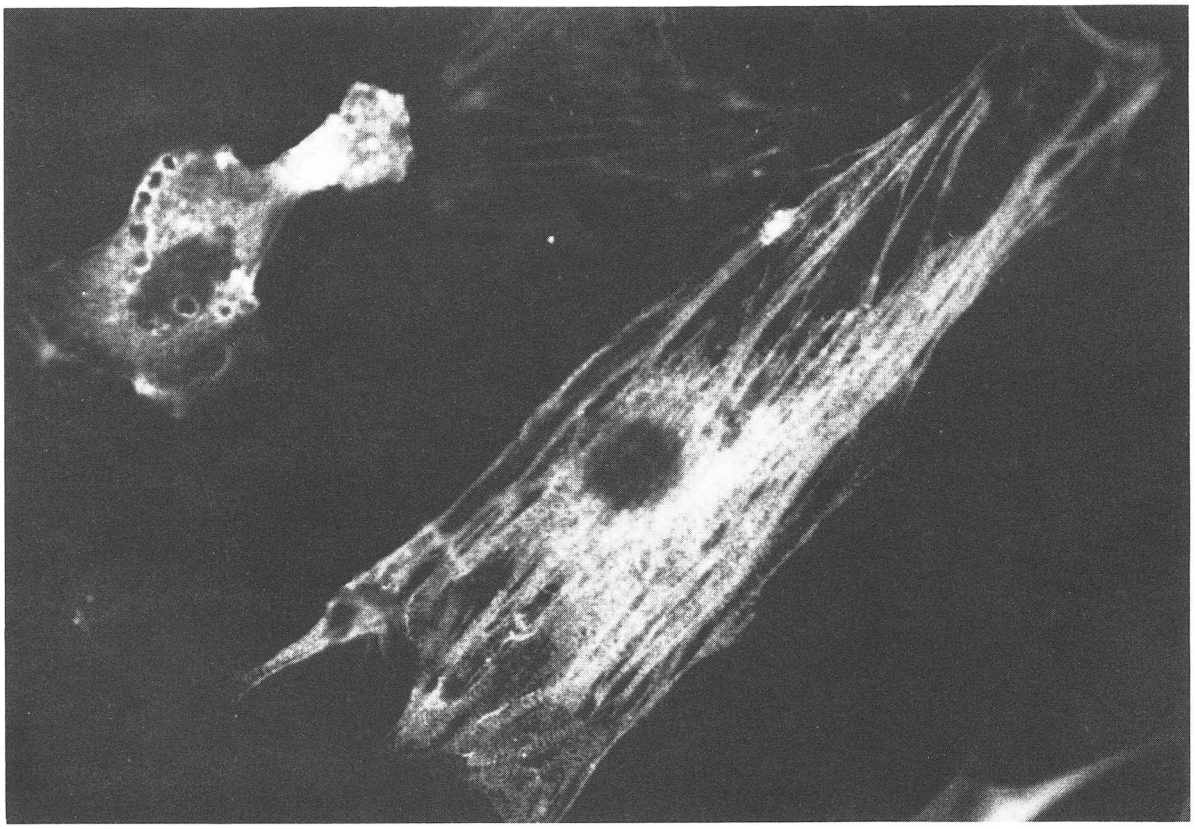

Fig.1. Cultured mesangial cells stained by indirect immunofluorescence using an anti-myosin antiserum, after incubation with 1 nM angiotensin II. Note the presence of intracellular fibrillar structures in one (non-contracting) cell and their absence in the other (contracting). This latter cell also exhibits membranous evaginations.

fluorescent technique. The cover slips were washed 3 times with phosphate-buffered saline during 3 min each time and mounted in 50% glycerin [4]. The cells were examined under fluorescence microscopy using a Leitz microscope equipped with an HBO 50 mercury lamp. About 500 cells were counted for each [A II] tested. Each experiment was performed in duplicate. Control mesangial cells incubated without A II were examined in parallel.

3. Results and discussion

3.1. *Binding studies*

^{125}I-A II binding was measured as a function of time using both epithelial and mesangial cells. Specific binding to epithelial cells was very low as compared with that obtained with mesangial cells. Therefore mesangial cells only were considered in the subsequent studies. The amount of bound ^{125}I-A II increased progressively with time and reached a steady state after 40 min incubation (fig.2). This experiment was

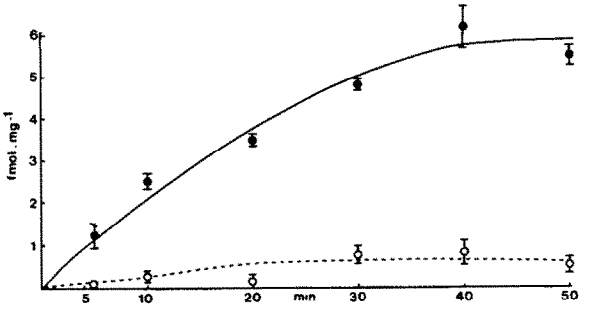

Fig.2. Binding of ^{125}I-angiotensin II (0.5 nM) to mesangial cells as a function of time: (•) total binding; (○) non-specific binding. Each point is the mean of triplicates and each vertical bar twice the SEM.

repeated at different [^{125}I-A II] to calculate the kinetic parameters of binding. The following equation was used:

$$\log_e RH_{eq}/(RH_{eq} - RH) = (k_{+1}\, h + k_{-1})t$$

where: RH = bound hormone at any time; RH_{eq} = bound hormone at equilibrium; h = hormonal concentration in the incubation milieu. If b_1 and b_2 are the slopes of two association curves studied at two different ^{125}I-A II concentrations (h_1 and h_2, respectively), the two following equations can be written: $b_1 = k_{+1}\, h_1 + k_{-1}$ and $b_2 = k_{+1}\, h_2 + k_{-1}$, from which k_{+1} and k_{-1} can be calculated. The values observed were $k_{-1} = 0.043\ min^{-1}$, $k_{+1} = 0.00246 \times 10^{10}\ M^{-1}.min^{-1}$ and $K_d = k_{-1}/k_{+1} = 1.77$ nM. Kinetic parameters could not be calculated from comparison between association and dissociation curves because, as in [11], the dissociation of the hormone–receptor complex did not obey first-order kinetics when Ca^{2+} and Mg^{2+} were present in the incubation medium. ^{125}I-A II specific binding was linearly related to the amount of cellular protein over 100–400 μg/ml (fig.3). This allowed the results to be expressed as

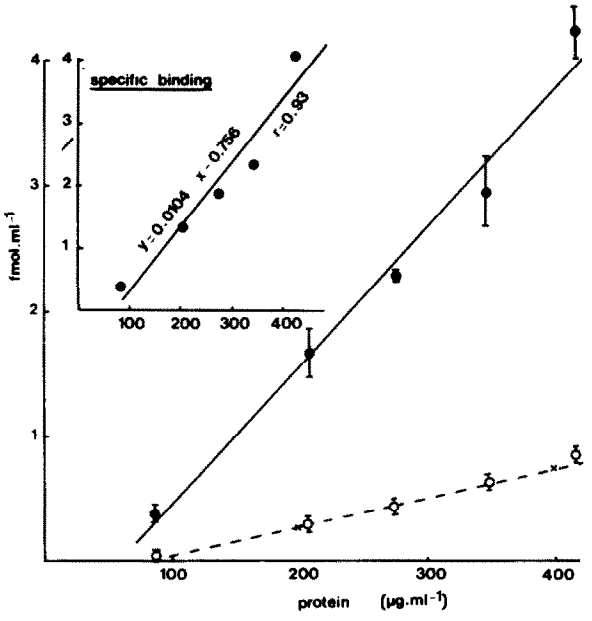

Fig.3. Binding of ^{125}I-angiotensin II as a function of the concentration of glomerular protein. Each point is the mean of duplicates and each vertical bar the difference between them: (•) total binding; (○) non-specific binding. Specific binding, obtained by subtracting the open from the closed circles, is given in the inset.

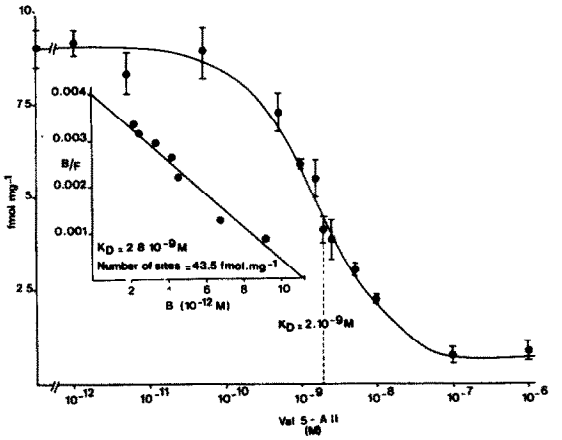

Fig.4. Competitive inhibition of binding of ^{125}I-angiotensin II to glomerular mesangial cells in the presence of increasing doses of unlabeled angiotensin II. Each point is the mean of duplicates and each vertical bar the difference between them. (---) The concentration corresponding to 50% of binding obtained without unlabeled hormone. The Scatchard transformation of the data obtained at 10^{-10}–10^{-8} M is given in the inset.

fmol A II bound/mg cellular protein. Dilution of ^{125}I-A II (0.5 nM) with increasing unlabeled [A II] from 1 pM–1 μM decreased the amount of labeled hormone bound to mesangial dissociated cells (fig.4). Residual binding >0.1 μM corresponded to non-specific binding and was <10% of maximum binding. The data in fig.4 were transformed according to Scatchard. A linear curve was obtained (fig.4, inset) corresponding to 1 group of receptor sites. The abscissa intercept allowed calculation of the no. sites (43.5 fmol/mg) and the slope, of the K_d value (2.8 nM). This latter value is close to that derived from the time-course studies (1.8 nM). The inhibitory potency of binding of various A II analogues was also studied. The results are shown on table 1. All the A II analogues tested at 1 μM strongly inhibited ^{125}I-A II binding. The effect of Sar^1, Ala^8 A II was identical to that of Asn^1, Val^5 A II. The other drugs were less potent. Unrelated peptides such as lysine vasopressin and the 1–34 fragment of bovine parathyroid hormone were entirely inactive.

These studies demonstrate that the binding sites for A II present in mesangial cultured cells possess most of the characteristics of hormonal receptors:

(i) Very low calculated K_d, ~1 nM, value similar to those measured for A II–receptor association in rat liver [12], adrenal cortex [13,14] and brain membranes [15];

Table 1
Competitive inhibition of binding of ^{125}I-angiotensin II to cultured mesangial cells

Drugs (1 μM)	^{125}I-Angiotensin II bound (fmol/mg)	Percentage of binding
None	5.04 ± 0.48[a]	100
Asn^1, Val^5 A II	1.08 ± 0.23	21.4 ± 4.6
Des Asn^1, Val^5 A II	1.52	30.1
Des Asp^1, Ile^5 A II	1.87	37.1
Sar^1, Ala^8 A II	1.03	20.4
Sar^1, Ile^8 A II	1.59	31.6
1–34 bovine PTH	5.07	100.5
Lysine-vasopressin	4.97	98.7

[a] The two first results were calculated from 6 individual values and are given as means ± SEM

The other results are means from duplicates

(ii) Specific inhibition of binding of labeled A II by unlabeled hormone and by analogs and antagonists;
(iii) Binding equilibrium with time.

In [16], we provided evidence for the presence of several groups of specific receptor sites for A II in glomeruli isolated from rat kidney cortex, and interpreted this finding as resulting from heterogeneity of the glomerulus and/or the multiplicity of A II effects on the glomerular function. Subsequently [3] we could localize some of these receptor sites on the glomerular basement membrane. This report indicates that another group of sites is present on the mesangial cells. ^{125}I-A II binding to mesangial cells was studied in the presence of Ca^{2+} and Mg^{2+} in the incubation medium at concentrations close to those present in the extracellular fluids, since it had been demonstrated that specific binding to isolated glomeruli was increased in this condition [11]. In agreement with these findings, the effects of A II on the parameters of glomerular filtration rate were reversed [17] when the rats were treated with verapamil, an inhibitor of calcium transport through the cell membranes. The concentration of A II receptor sites appears to be clearly smaller in cultured mesangial cells than in isolated whole glomeruli incubated in similar conditions [11]. This could reflect either a loss of receptors during cultures or binding to only a subpopulation of cultured cells.

3.2. *Degradation studies*

Degradation of ^{125}I-A II in the presence of dissociated mesangial cells was measured with the techniques of rebinding to fresh glomeruli and binding to an excess of specific antibodies (table 2). The first of these techniques gave % values of degraded hormone greater than the second one. Anyway the amount of degraded hormone was ⩽25% after 50 min incubation. These results are similar to those observed with isolated glomeruli [1,3,11,16].

Table 2
Degradation of ^{125}I-angiotensin II present in the incubation medium

Incubation (min)	Binding to an excess of antibody[a]	Rebinding to fresh glomeruli[a]
10	4.5%	13.5%
20	4.5%	26.5%
50	11%	24.4%

[a] Each point is the mean of duplicates

3.3. *Cell reactivity studies*

After incubation in the Hank's balanced salt solution containing 2 mM Ca^{2+} and 2 mM Mg^{2+}, the mesangial cells were frequently surrounded by a prominent contrast halo due to a greater retractility and presented either elongated, fusiform or spherical shapes [9]. After incubation with A II, changes in length or shape of the mesangial cells were observed within a few minutes and progressed until ~15 min after addition of this hormone [9]. Similarly, the myosin distribution in the contracting mesangial cells was quite different from that observed in the non-contracting ones (fig.1). The percentage of contractile cells increased progressively with the concentration of A II added and reached a steady value >10 nM. A sigmoidal effect *vs* $\log_{10}$ dose–curve (was obtained fig.5). The calculated ED_{50} ([A II] corresponding to 50% of the maximum effect) was 0.74 nM. This value is close to the K_d values obtained from time-course studies or competitive inhibition studies (fig.4). This suggests a specific relationship between binding sites for A II and mesangial cell contractility. Other arguments in favor of this conclusion are the parallel stimulatory effect of Ca^{2+} and the parallel inhibitory effects of (Sar^1, Ala^8) A II on ^{125}I-A II binding [11] and cell reactivity [9]. A maximum of 32% was reached for the % of contractile cells. Here, we could not determine whether or not ^{125}I-A II binds only to those cells which contract. An affirmative answer is suggested by the low concentration of A II receptor

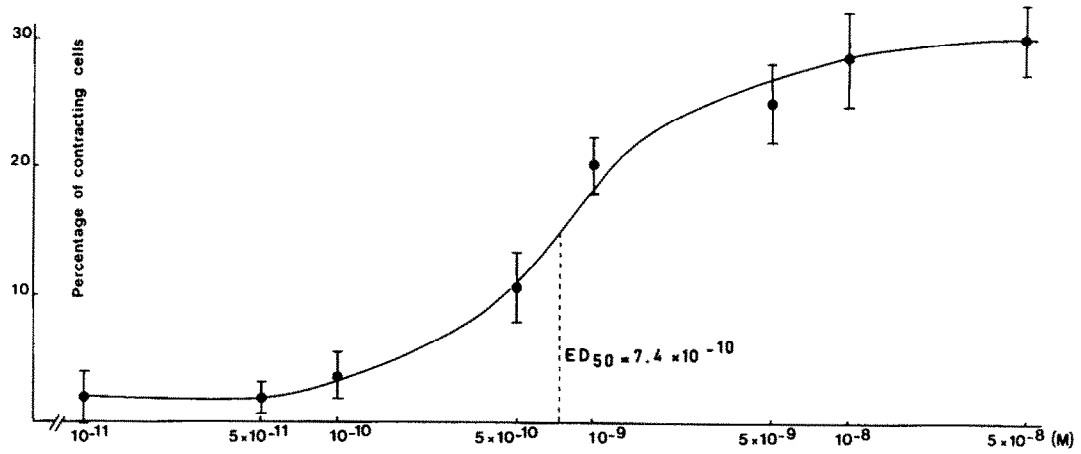

Fig.5. Percentage of contractile mesangial cells after 15 min incubation with unlabeled angiotensin II plotted against [angiotensin II]. Each point represents the mean of 3 determinations and each vertical bar twice the SEM. For each determination, ~500 mesangial cells were counted. (---) The concentration (ED_{50}) corresponding to 50% of the maximum effect.

sites compared with that observed with whole glomeruli. Contraction of mesangial cells in the presence of 1 nM A II was also observed in [5] but similar behavior for all the cells examined was reported. This discrepancy is probably related to the fact that cloned mesangial cells were used in [5] but not in ours. Cloned cells which derive from the same single cell exhibit identical biological properties when they are tested. In [5] arginine vasopressin at 0.1 nM also stimulated mesangial cell contractility and ^{3}H-labeled lysine vasopressin specifically bound to the glomerular mesangial cells with an app. K_d of 10 nM. The binding studies were, however, limited to time-course experiments.

4. Conclusion

These findings clearly indicate the presence of specific receptors for A II in mesangial cultured cells of glomerular origin and suggest that binding to these receptors may be the first step of the effect of A II on glomerular vasoreactivity through contraction of the mesangial cells. Specific receptors for A II were probably also present in epithelial cells but at a much lower concentration. One may therefore consider that the high affinity binding sites for A II represent a biochemical marker of the mesangial cells. Furthermore, since the mesangial cells bind A II, contract in the presence of A II, and produce large amounts of PGE_2 [6] it seems reasonable to suggest that these cells, and not the epithelial cells, play a major role in the control of glomerular vasoreactivity. It is also possible that A II binding sites are related to PG production. However, the stimulation of PG synthesis by the mesangial cells in the presence of A II is only clear at ~10 nM [18] which are higher than those required for specific binding here.

Acknowledgements

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