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Evidence for angiotensin IV receptors in human collecting duct cells

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Evidence for angiotensin IV receptors in human collecting duct cells. Because angiotensin II (Ang II) has been found at high concentrations in the proximal tubule fluid and because tubular brush border membranes exhibit a marked capacity for degrading Ang II, we thought it of interest to examine the binding sites for Ang II (3-8) (referred to as Ang IV), a metabolite of Ang II, downstream in the nephron. We studied the binding of [^{125}I]-Ang IV and also of [^{125}I]-Sar^1, Ala^8, Ang II to SV-40 transformed human collecting duct cell (HCD) membranes. No specific binding site for [^{125}I]-Sar^1, Ala^8, Ang II and no Ang II-dependent cytosolic calcium response could be observed. Moreover, no signal for the human type I Ang II receptor (hAT$_1$) mRNA was present in HCD cells. In contrast, [^{125}I]-Ang IV bound specifically to HCD cell membranes. Mean K_d and B_{max} values derived from saturation binding studies were 5.6 ± 2.0 nM and 1007.6 ± 140.2 fmol/mg protein, respectively. The rank order of affinity for competitive Ang II-related peptides was: Ang IV > Ang III > Ang II > Ang II (4-8) > Ang II (1-7). [^{125}I]-Ang IV binding was not modified by nonpeptide AT$_1$ (losartan) or AT$_2$ (PD123177) antagonists. GTPγS and dithiotreitol did not affect [^{125}I]-Ang IV binding. Ang IV stimulated cAMP production by intact HCD cells in the presence of forskolin but did not modify cGMP production or cytosolic calcium concentration. Taken together, these results indicate that HCD cells represent a target site for Ang IV but do not possess Ang II receptors.

Recent evidence indicates that the angiotensin II (Ang II) C terminal hexapeptide, also designated as angiotensin IV (Ang IV), is not an inactive metabolite but possesses the characteristics of a real hormone with specific receptors and biological effects [1]. The kidney, which is one of the main target sites for Ang II, is also rich in enzymes catabolizing this hormone including aminopeptidases, carboxypeptidases and neutral endopeptidase [2, 3]. Such enzymes are present in high density at the luminal surface of the proximal tubule cells. Moreover, it has been shown that the concentration of Ang II in the proximal tubule fluid is high (10 nM) reaching 500 to 1000 times that simultaneously observed in the plasma [4, 5]. It is thus likely that high amounts of Ang IV are formed in this part of the nephron. Collecting duct cells play a major role in the final adjustment of the sodium balance. Their principal cells possess aldosterone and arginine vasopressin receptors that control the activity of the amiloride-sensitive sodium channel [6]. Early micropuncture studies suggested that Ang II might inhibit sodium reabsorption in the distal tubule [7]. Binding studies on microdissected nephron segments demonstrated that Ang II bound specifically to the rat cortical and inner medullary collecting duct [8]. These studies could not distinguish the Ang II receptor subtype. More recently, Zhuo et al, using high resolution light and electronic autoradiography, did not detect any [^{125}I]-Sar^1, Ala^8, Ang II binding on the rat collecting duct after injection of this tracer into the renal artery [9]. In contrast, Terada et al, using the reverse transcription-polymerase chain reaction technique in individual microdissected rat renal tubule segments, found a marked signal for the type I Ang II (AT$_1$) receptor in the cortical collecting duct. Smaller but detectable bands were observed in the outer and the inner medullary collecting ducts [10]. Our aim was to characterize the binding sites for Ang II and its metabolite, Ang IV, in the principal cells of the human collecting duct. Whereas no demonstration of the presence of specific Ang II receptors could be made, we provided evidence for high-affinity Ang IV binding sites with all the characteristics of the Ang IV receptor previously described in other tissues [1, 11, 12].

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Methods

Materials

Reagents for these studies were obtained as follows: [^{125}I]-Sar^1, Ala^8-Ang II and [^{125}I]-Ang IV (74 TBq/mmol) from the "Centre d'Etudes Nucléaires" (Gif-Sur-Yvette, France); [^{3}H]-myoinositol (2.7 TBq/mmol), [^{125}I]-cyclic AMP, [^{125}I]-cyclic GMP, anti-cyclic AMP antibody and anti-cyclic GMP antibody from The Radiochemical Centre (Amersham, UK); culture media, antibiotics and cell culture supplies from Gibco (Paisley, UK); newborn calf serum (NCS) from Bochringer (Mannheim, Germany); ionomycin and fura-2-acetoxymethyl ester from Calbiochem (San Diego, CA, USA); Ang II (3-8) (also referred to as Ang IV), Ang II (4-8), Ang II (1-7) and human atrial natriuretic factor (ANF) from Peninsula (London, UK). Ang II was a gift from Ciba-Geigy (Basel, Switzerland). Losartan and its metabolite, EXP 3174, two nonpeptide AT$_1$ antagonists, were donated by Merck, Sharp and Dohme Research Laboratories (West Point, PA, USA); CV 11974, another AT$_1$ antagonist, and PD 123177, an AT$_2$ antagonist, were donated by Takeda (Tokyo, Japan) and Parke-Davies (Ann Arbor, MI, USA), respectively. All other reagents were from Sigma (St. Louis, MO, USA).

Cell culture

The human collecting duct cell line (HCD) was established previously by transfection of a human tubular cell suspension with a replication-defective strain of SV40 virus. This cell line has been previously identified and expresses the essential characteristics of the principal cells [13]. Cells were regularly cultured at 37°C in an atmosphere of 5% CO_2-95% air in a hormonally-defined medium (DMEM-HAM's F12, 1:1 (vol/vol); transferrin, 5 μg/ml; sodium selenate, 50 nM; glutamine, 2 mM; dexamethasone, 50 nM; insulin, 5 μg/ml; HEPES, 20 mM, pH 7.4) containing 2% NCS. They were seeded at a density of 10,000 cells/cm^2 in 12-well plates or 100 mm Petri dishes. Cultures were re-fed with fresh medium every two to three days. All the experiments presented in this study were performed on confluent cells from passages 20 to 47 that had been cultured for five days.

Cell membrane preparation

Membranes of HCD cells were prepared according to the technique utilized by Hall et al for cultured vascular smooth muscle cells with minor modifications [11]. HCD cells grown at confluence were washed once at 37°C in phosphate-buffered saline (in mM: NaCl, 150; Na_2HPO_4, 9.2; NaH_2PO_4, 2.3; pH 7.4). This was followed by the addition to the culture dish of 2 ml of cold (4°C) isotonic buffer (in mM: NaCl, 150; Tris, 50; EDTA, 1; phenylmethylsulfonyl fluoride, 1; bestatin, 0.01; pH 7.4). Cells were then removed from the plates with a rubber policeman and homogenized in 5 ml of assay buffer for approximately 10 seconds (Polytron, Brinkman Instruments). The homogenate was centrifuged at 40,000 g for 20 minutes at 4°C, the supernatant was discarded, the pellet was rehomogeneized in the buffer and the centrifugation was repeated. The final pellet was resuspended in the assay buffer to a working concentration of approximately 1 mg protein/ml as determined by the method of Lowry et al [14]. The membrane suspension was utilized immediately following preparation.

Binding studies

Membrane receptor binding assays were performed using [^{125}I]-Sar^1, Ala^8, Ang II or [^{125}I]-Ang IV according to Hall et al with minor modifications [11]. Duplicate samples were incubated at 37°C under continuous shaking for 120 minutes with 0.4 nM of [^{125}I]-Ang IV or 0.9 nM of [^{125}I]-Sar^1, Ala^8, Ang II in a total volume of 250 μl of assay buffer (NaCl, 150 mM; Tris, 50 mM; EDTA, 1 mM; PMSF, 1 mM; bestatin, 20 μM; BSA, 0.1%; pH 7.4) containing 100 μg of membrane protein. Bound and free ligands were separated at the end of each experiment by addition of 4 ml of ice-cold phosphate-buffered saline and vacuum filtration through a Whatman GF/B filter followed by three additional washes (4 ml) with phosphate-buffered saline. The [^{125}I] radioactivity retained by the filters was counted in a LKB (Malmoe, Sweden) gamma counter with 60% counting efficiency. Kinetic studies, competitive binding experiments and saturation binding experiments were performed. The binding parameters, K_d and B_{max}, were derived from the Scatchard's transformation of the data obtained in the latter two study types. Nonspecific binding was measured in the presence of 1 μM unlabeled ligand and specific binding was calculated as the difference between total and nonspecific binding. It was expressed as femtomoles of [^{125}I]-Ang IV or [^{125}I]-Sar^1, Ala^8, Ang II bound per mg of protein.

For G-protein linkage experiments, the membrane preparations were first preincubated at 22°C for 60 minutes in the assay buffer supplemented with 5 mM $MgCl_2$ and containing increasing concentrations of GTP-γS (0.1 to 100 μM). Pretreated membranes were then studied in equilibrium binding experiments.

Binding studies were also performed using intact cells in monolayers. Confluent cells in 12-well plates were rinsed three times with 0.15 M NaCl. Then they were incubated at 22°C for 30 minutes with [^{125}I]-Ang IV (0.15 nM) in 500 μl of buffer (NaCl, 135 mM; Tris, 20 mM; glucose, 5 mM; KCl, 10 mM; $NaCH_3COO$, 10 mM; $CaCl_2$, 2 mM; BSA, 0.2%; pH 7.4) in the presence of increasing concentrations of unlabeled Ang IV (1 nM to 10 μM) or of various aminopeptidase N (APN) inhibitors. At the end of the incubation period, the medium was removed and the cells were rinsed three times with 2 ml of ice-cold 0.15 M NaCl. The cells were then dissolved in 1 M NaOH and [^{125}I] radioactivity was counted.

Enzyme assays

APN activity was measured on cell membranes as previously described [15]. Membranes (100 μg) were suspended in 0.5 ml of Ca^{2+}-free phosphate-buffered saline additioned with 1 mM $MgCl_2$. The enzymatic reaction was started by addition of 1 mM alanine-p-nitroanilide as substrate. Incubation was carried out with gentle agitation for 5 to 20 minutes under zero-order kinetic conditions. The amount of p-nitroanilide formed was measured in the supernatant at an O.D. of 405 nm. Cell-free and substrate-free blanks were run in parallel. Enzyme activity was expressed as nmol p-nitroanilide formed per min per mg of protein.

Cyclic AMP and cyclic GMP assays

HCD cells grown at confluence in 12-well trays were incubated for 15 minutes at 37°C in their culture medium in the presence of 0.1 mM 3-isobutyl-1-methyl xanthine (IBMX), a phosphodiesterase inhibitor. The medium was then discarded and replaced by the same medium with or without the agents to be tested: Ang IV (0.1 μM) and forskolin (100 μM), alone or in combination for cyclic AMP, Ang IV (0.1 μM) and ANF (0.1 μM), alone or in combination for cyclic GMP. After a five minute incubation at 37°C, the medium was quickly recovered and the reaction was stopped by adding 0.5 ml of ice-cold 0.1 M HCl. After 10 minutes at 4°C, the supernatant was collected and added to the medium. Only the resulting specimens for cyclic GMP determination were acetylated. Then all specimens were diluted in 50 mM sodium acetate buffer, pH 6.2. Cyclic nucleotides were measured by radioimmunoassays using specific anti-cyclic AMP and anticyclic GMP antibodies. Results were expressed as pmol/mg/5 min.

Inositol phosphate analysis

Inositol phosphates (IPs) were measured as described [16]. After the cells had reached subconfluency, culture medium was removed and cells were placed in inositol-deficient Waymouth medium containing 1.1 MBq/ml of [^{3}H]-myoinositol (2 ml per well) for 48 hours at 37°C. After 15 minutes preincubation with 10 mM of lithium chloride, 0.1 μM of Ang IV was added. A positive control was performed with bradykinin at the same concentration. The incubation was terminated by quickly aspirating the medium and adding 2 ml of ice-cold 5% trichloroacetic acid. Cells were scraped away from the wells, washed once more with 5% trichloroacetic acid and the aqueous phase was extracted in diethylether.

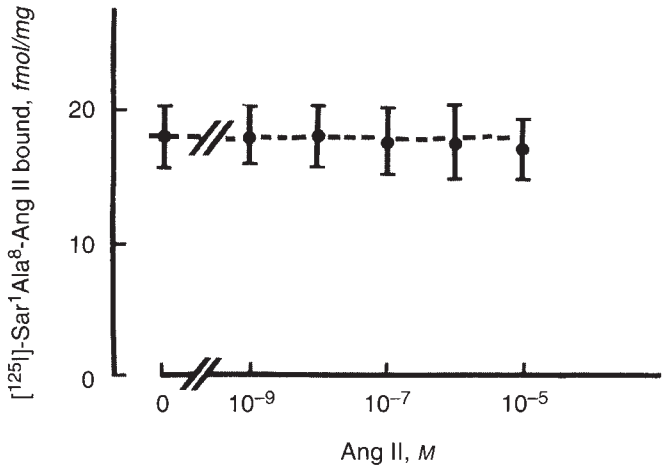

Fig. 1. *[^{125}I]-Sar^1, Ala^8, Ang II binding to HCD cell membranes in the presence of increasing concentrations of Ang II.* Data are means ± SE of 3 individual experiments (each with duplicate samples) are shown.

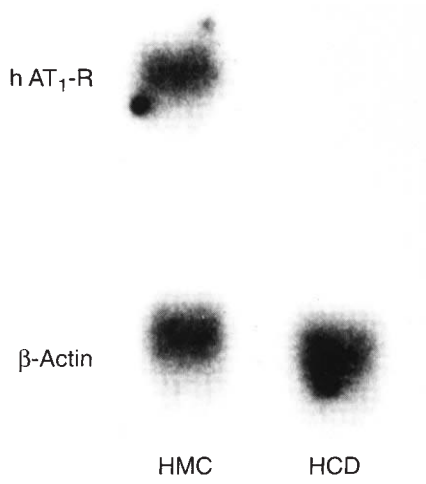

Fig. 2. *Northern blot analysis showing that hAT$_1$ mRNA is expressed in human mesangial cells (HMC) but absent in HCD cells.* β-actin mRNA was used as a control.

Samples were adjusted to pH 7.0 with 50 mM of sodium tetraborate and then loaded into 2 ml Dowex AG1-X8 anion exchange resin columns (Biorad). The columns were washed with 10 ml of water and 10 ml of 5 mmol/liter sodium tetraborate. IPs were then eluted with 10 ml of increasing concentrations of ammonium formate in 0.1 mM formic acid. Five milliliters of each collected fraction were mixed with scintillation fluid and counted in a beta counter (LKB).

Cytosolic free calcium determination

Cells were cultured on thin glass microscope coverslips precoated with 0.2% gelatin and were studied at subconfluence. Cells were loaded with 1.5 μM Fura 2/AM at 37°C for 90 minutes. For measurements of fluorescence, each coverslip was placed on the stage of the inverted microscope and one cell was selected. The sample was then superfused at a rate of 0.6 ml/min at 37°C with basal medium or with the solution to be tested. Fura 2 was alternatively excited at wavelengths of 340 and 380 nm using a 75-W xenon light source, filters, and a chopper (PTI Photoscan II System; Kontron). The fluorescence intensities (S at 340 nm and L at 380 nm) issued from the selected cell and delimited by means of an adjustable window were measured. $[Ca^{2+}]_i$ was calculated from the following equation: $[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)](L_{max}/L_{min})$ where K_d = 224 nM, R = S/L, and L_{min}, L_{max}, R_{min} and R_{max} are L and R values at 0 and saturating concentrations of calcium, respectively. L_{max}, L_{min}, R_{min} and R_{max} were determined by external calibration as previously described [17].

Preparation of RNA and Northern analysis of hAT$_1$ receptor

Total RNA from human collecting duct cells was extracted by the phenol-chloroform method and precipitated with 3 M LiCl [18]. RNA concentration and purity were determined from the absorbance readings at 260 and 280 nm. Total RNA was then fractionated by electrophoresis in agarose gel and transferred to a Nylon Genescreen Plus membrane (New England Nuclear, Boston, MA, USA). After prehybridization, the blot was hybridized for 16 hours at 42°C with an [α^{32}P]-labeled cDNA probe specific for the human AT_1 receptor [19]. Total RNA from human mesangial cells was used as a positive control [20].

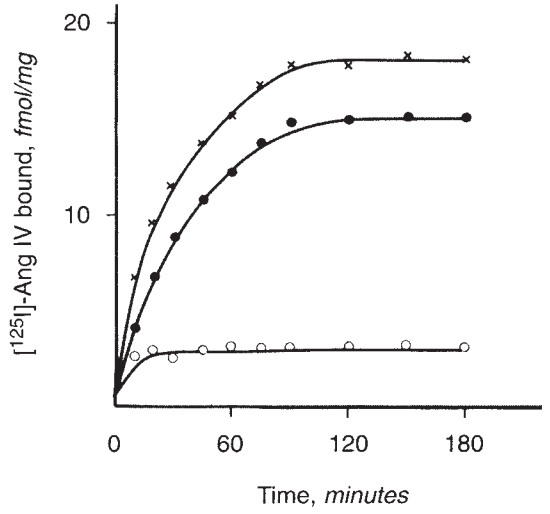

Fig. 3. *Time-course study of [^{125}I]-Ang IV binding to HCD cell membranes.* A representative experiment is shown. Each point is the mean of duplicates. Specific binding was calculated as the difference between total and nonspecific binding.

After hybridization, the filters were washed three times at 42°C for 20 minutes in 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) containing 0.1% sodium dodecylsulfate (SDS) and exposed to a Fuji X-ray film at −80°C in the presence of intensifying screens. The same filter was dehybridized by boiling for 15 minutes in 0.1 × SSC containing 1% SDS and rehybridized with a [^{32}P]-labeled β-actin cDNA probe.

Results

Binding studies of [^{125}I]-Sar^1, Ala^8, Ang II on HCD cell membranes and absence of hAT$_1$ mRNA expression in HCD cells

Our initial aim was to examine whether or not there were specific binding sites for Ang II at the surface of HCD cells. Therefore, we studied [^{125}I]-Sar^1, Ala^8, Ang II binding to HCD cell membranes. Only nonspecific binding sites could be detected

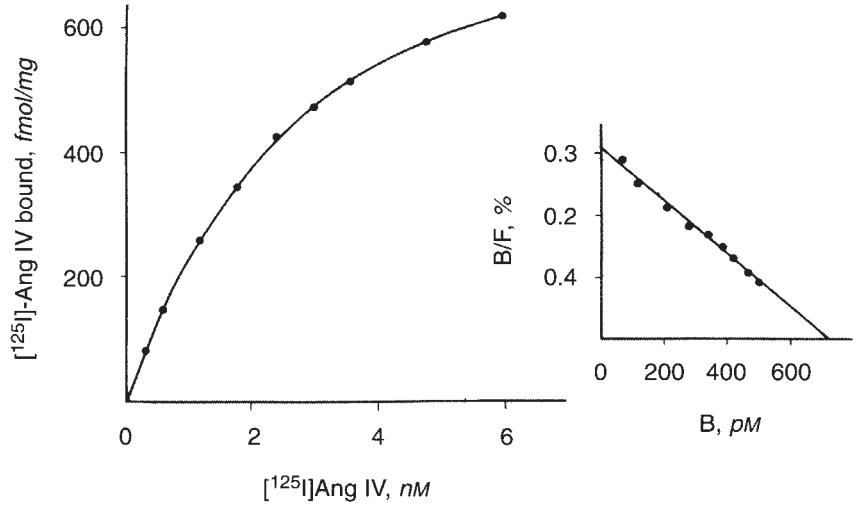

Fig. 4. *Saturation binding study of [^{125}I]-Ang IV to HCD cell membranes.* One representative experiment is shown. Each point is the mean of duplicates. The Scatchard's transformation of the data are shown in the inset (K_d = 2.3 nM; B_{max} = 894.4 fmol/mg). Two additional studies were performed.

as shown by the absence of displacement of the bound tracer even at high concentrations of Ang II (Fig. 1). We also examined whether or not hAT_1 mRNA was expressed in HCD cells. The Northern blot technique using a human AT_1 cDNA specific probe did not reveal any signal. Positive controls were obtained in the same experiment with human mesangial cells (Fig. 2). These initial results led us to study the binding of Ang IV which could have been formed locally after cleavage of the two N-terminal amino acids of the tracer.

Binding studies of [^{125}I]-Ang IV on HCD cell membranes

The results observed differed substantially from those obtained with [^{125}I]-Sar^1, Ala^8, Ang II. Kinetic studies showed that [^{125}I]-Ang IV binding to cell membranes reached a plateau after 90 minutes incubation that persisted up to 180 minutes. At equilibrium, nonspecific binding represented 15% of total binding (Fig. 3). Saturation binding experiments were performed at equilibrium after 120 minutes incubation. The amount of [^{125}I]-Ang IV specifically bound increased curvilinearly as a function of [^{125}I]-Ang IV concentration in the medium. Scatchard's transformation of the data revealed that there was a single group of receptor sites (Fig. 4). The mean K_d and B_{max} values obtained from three experiments were 5.6 ± 2.0 nM and 1007.6 ± 140.2 fmol/mg membrane protein. Competitive inhibition of binding of [^{125}I]-Ang IV to HCD cell membranes at equilibrium in the presence of increasing concentrations of Ang II-related peptides indicated a high specificity for Ang IV (Fig. 5). Scatchard's transformation of these data that had been obtained over a large range of concentrations provided results in the same range as those found in saturation binding experiments. There was a single group of receptors, and mean K_d and B_{max} values derived from three experiments were 9.0 ± 0.78 nM and 1368 ± 350 fmol/mg. The rank order affinity of competitive Ang II-related peptide was as follows: Ang IV > Ang III > Ang II > Ang II (4-8) > Ang II (1-7). [^{125}I]-Ang IV binding to HCD cell membranes was not affected by nonpeptide AT_1 (losartan) or AT_2 (PD123177) antagonists (Fig. 6). Other AT_1 antagonists (EXP 3174 and CV 11974) were tested with the same negative results (results not shown). These data indicated that the Ang IV binding sites present on the cell membranes did not correspond to classical AT_1 or AT_2

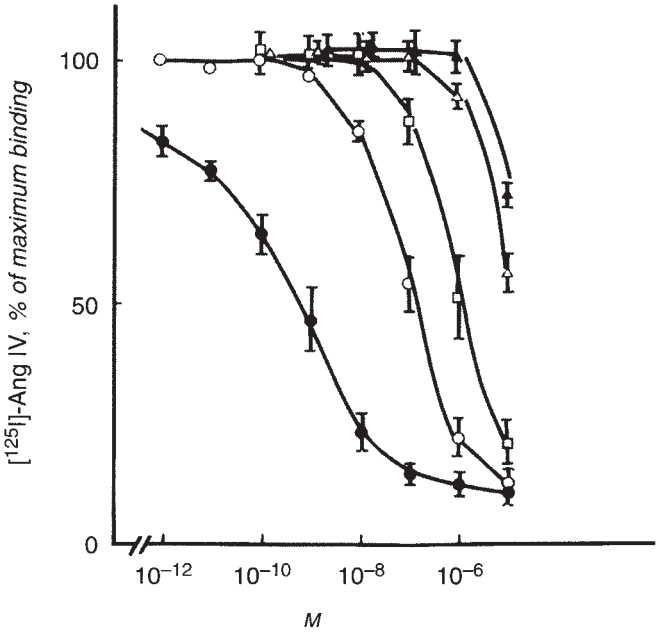

Fig. 5. *Competitive inhibition of binding of [^{125}I]-Ang IV to HCD cell membranes in the presence of increasing concentrations of Ang IV (●), Ang III (○), Ang II (□), Ang II (4-8) (△) and Ang II (1-7) (▲).* Data are the means of 2 to 4 individual experiments (each with duplicate samples).

receptors. They also were distinct from APN, one of the main enzymes hydrolyzing Ang III and Ang IV [21, 22], since binding studies were performed in the presence of an excess of bestatin, an APN inhibitor. A non-hydrolysable GTP analogue, GTP-γS, did not affect the [^{125}I]-Ang IV binding to HCD cell membranes over a large range of concentrations (0.1 to 100 μM). Negative results were also obtained in the presence of increasing concentrations of dithiotreitol (0.01 to 10 mM). The latter results confirmed that the binding sites studied were distinct from the AT_1 receptors.

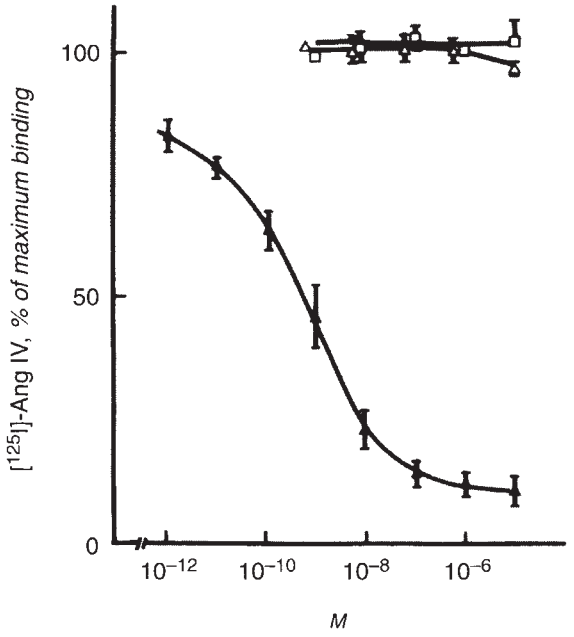

Fig. 6. *Lack of competitive effect of losartan (□), an AT_1 antagonist, and of PD123177 (△), an AT_2 antagonist, on [^{125}I]-Ang IV binding to HCD cell membranes.* The competitive inhibition curve in the presence of unlabeled Ang IV (▲) is also shown. Data are the means of 2 to 3 individual experiments (each with duplicate samples).

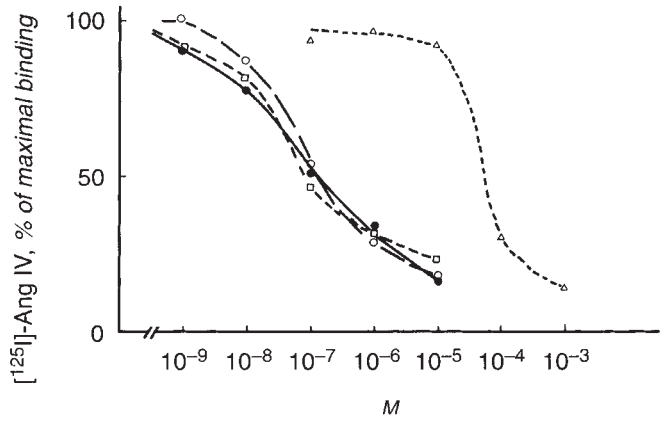

Fig. 7. *Competitive inhibition of binding of [^{125}I]-Ang IV to HCD cells in the presence of increasing concentrations of unlabeled Ang IV (●), bestatin (○), amastatin (□), and 1,10 phenantroline (△).* Intact cells in monolayers exposing their apical poles to the medium were studied. Data are the means of 3 individual experiments (each with duplicate samples).

Binding studies of [^{125}I]-Ang IV on intact HCD cells

[^{125}I]-Ang IV specifically bound to HCD cells as shown by the marked displacement of the tracer (85%) obtained in the presence of an excess of unlabeled Ang IV (Fig. 7). Unexpectedly, bestatin and amastatin, two competitive inhibitors of aminopeptidase N activity, were as potent competitors as unlabeled Ang IV with a 50% inhibitory concentration (IC_{50}) of approximately 0.1 μM for the three agents. A noncompetitive inhibitor of aminopeptidase N, 1,10 phenanthrolin, also exhibited an inhibitory potency but at a much lesser degree (IC_{50} = 56.4 μM).

Biological effects of Ang IV on intact HCD cells in monolayer

Ang IV (100 nM) did not modify intracellular calcium concentration $[Ca^{2+}]_i$ or inositol phosphate formation, thus indicating that Ang IV did not stimulate the phospholipase C pathway. Positive controls were obtained in both cases with bradykinin (100 nM). We also verified that Ang II was inactive, which confirmed the absence of AT_1 receptors in HCD cells. The results of $[Ca^{2+}]_i$ determinations are shown in Figure 8. The only biological effect observed was the Ang IV-stimulated cyclic AMP production in the presence of 100 μM forskolin (261 ± 33 and 178 ± 19 pmol/mg/5 min with and without 0.1 μM Ang IV, respectively; N = 6, $P < 0.05$). In contrast, Ang IV did not enhance basal cyclic AMP formation (22 ± 4 and 23 ± 5 pmol/mg/5 min with and without Ang IV, respectively). Ang IV also did not affect cyclic GMP production in HDC cells (0.35 ± 0.05 and 0.40 ± 0.06 pmol/mg/5 min with and without 0.1 μM Ang IV, respectively). A positive control was obtained with 0.1 μM ANF (112.0 ± 20.1 pmol/mg/5 min). Ang IV also did not modify the ANF-dependent cyclic GMP concentration (104.1 ± 21.2 pmol/mg/5 min).

Enzyme studies in HCD cell membranes

APN activity was detected in HCD cell membranes at a level of 17.9 ± 4.0 nmol/min/mg (N = 4). Bestatin inhibited APN activity at the concentrations present in the medium for membrane preparation and in the incubation medium (10 and 20 μM, respectively).

Discussion

We report in the present study that a human renal cell line deriving from the cortical collecting duct and expressing principal cell characteristics [13] possesses specific receptors for Ang IV. In contrast, no Ang II receptor could be demonstrated.

Evidence for Ang IV specific receptors on HCD cell membranes is supported by the following findings: (1) [^{125}I]-Ang IV bound to cell membranes in a specific saturable manner. Equilibrium of binding occurred after 90 minutes of incubation and nonspecific binding represented only 15% of total binding when the plateau had been reached. There was a single group of receptor sites with a K_d of approximately 5 nM. This value is in accordance with those reported by others [11, 23]. Competitive inhibition experiments confirmed the high affinity of the receptor for Ang IV with a lesser inhibitory potency for the other Ang II-related peptides. The rank order of potency was identical to that previously reported for Ang IV receptors in other tissues including bovine adrenal cortical membranes [1], bovine vascular smooth muscle cells [11] and cultured rabbit cardiac fibroblasts [23]. In particular, Ang II was 1,000 times less potent than Ang IV in the displacement of [^{125}I]-Ang IV from its receptor. (2) Ang IV binding sites did not recognize AT_1 (losartan, EXP 3174, CV 11974) and AT_2 (PD 123177) antagonists. (3) [^{125}I]-Ang IV binding was neither affected by GTP-γS nor by dithiotreitol, confirming that the binding sites studied were different from the two classical Ang II receptor subtypes. The lack of effect of GTPγS suggesting non-G-protein linkage is in agreement with other reports [12, 23]. In contrast, a stimulatory effect of dithiotreitol on Ang IV binding to bovine adrenal cortex membranes has been reported by Jarvis and Gessner [24]. (4) Ang IV stimulated cyclic AMP production by HCD. However, the degree of stimulation was low and activation of the catalytic site by

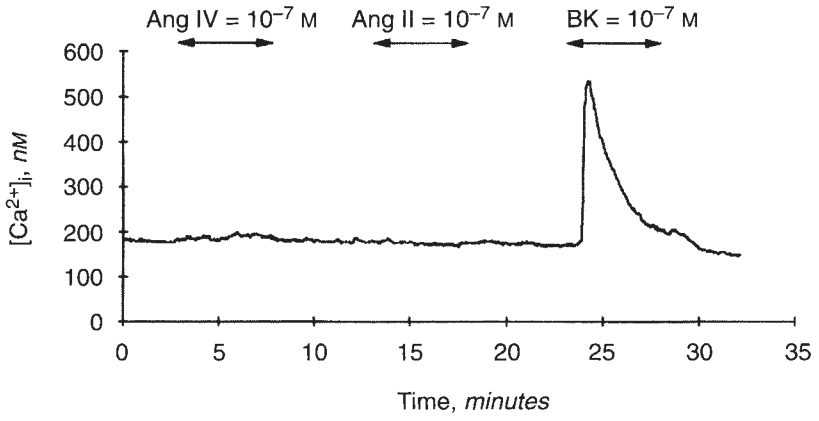

Fig. 8. *Representative single cell tracing of the $[Ca^{2+}]_i$ response to Ang IV, Ang II and bradykinin (0.1 μM each) in HCD cells.* Times of application of these agents are indicated by the corresponding lines.

forskolin was necessary to obtain a response. Such a combination of forskolin and hormones has been previously used to reveal cyclic AMP responses to hormones that are low or not observed in the absence of forskolin. It is generally admitted that forskolin through its synergistic interaction with hormones acts to increase a small hormone-dependent response that is already present in the unresponsive cells [25]. (5) Ang IV binding sites are not hydrolyzing enzymes, in particular not APN, since bound [^{125}I]-Ang IV was not displaced by an excess of bestatin, an APN inhibitor, which was regularly present in the incubation medium.

To determine the cellular localization of Ang IV receptors, we studied the binding of [^{125}I]-Ang IV to intact HCD cells in monolayers that exposed their apical membranes to the incubation medium. [^{125}I]-Ang IV binding to intact cells was completely displaced by APN inhibitors, whereas opposing results were obtained using cell membranes, strongly suggesting that the receptors for Ang IV are located mainly at the basolateral pole of the HCD cells. The binding sites present on the apical membrane are likely to be enzyme proteins, in particular APN, since their affinity for Ang IV was the same as that of two APN competitive inhibitors, bestatin and amastatin. However, this affinity was lower than that of Ang IV for the specific receptor identified on the membrane preparation, since 50% of maximum binding of [^{125}I]-Ang IV was displaced by 1 nM of Ang IV on the membranes and 100 nM of Ang IV on the cells. This finding is in agreement with the generally accepted view that hormones have a greater affinity for their receptors than for their hydrolyzing enzymes. The exclusive location of APN at the apical side has already been reported in cultured tubular cells [26]. However, even in small numbers, Ang IV specific receptors should be present in intact cells on their luminal membranes, since by using this preparation an increase in cyclic AMP in the presence of Ang IV and forskolin was observed. Such a hypothesis would be in agreement with the intraluminal formation of Ang IV from Ang II via the successive actions of APA and APN that are present all along the nephron [27, 28], and also with the previously published report of Dulin et al [29] showing Ang IV receptors both on the basolateral membranes and the brush border membranes purified from the rabbit renal cortex. Alternatively, as recently observed in cultured bovine adrenal medullary cells [30], Ang II could be converted into Ang IV by an endosomal pathway. However, this would require an internalization of Ang II, which is unlikely due to the absence of Ang II receptors on HCD cells.

Ang IV specific binding sites in the kidney have been previously localized to the outer stripe of the medulla in the rat using autoradiographic analysis [30]. They are also present in the guinea pig and the monkey kidney [1, 31]. More precisely, Dulin et al demonstrated specific binding of Ang IV to rabbit basolateral membranes and brush border membranes of the renal cortex [29]. Ang IV in contrast with Ang II increases cortical blood flow [1]. It also stimulates DNA and RNA synthesis in cultured rabbit cardiac fibroblasts [23]. The role of Ang IV specific receptors in the human collecting duct remains unknown. Stimulation of cyclic AMP in HCD cells by Ang IV could influence water and ionic transports that are sensitive to cyclic AMP concentration through protein kinase A activation and channel protein phosphorylation. Indeed, cyclic AMP activates apical Na^+ entry in the collecting duct which results, via an increase in intracellular sodium concentration, in the stimulation of the basolateral Na^+/Ca^{2+} exchanger [32].

Finally, we could not find any evidence for the presence of Ang II receptors on HCD cells. There was no specific binding of Sar^1, Ala^8, Ang II to cell membranes and no cytosolic calcium response after exposure of the cells to 0.1 μM of Ang II. Furthermore, no hAT_1 mRNA signal was found using the Northern blot technique. It is possible that the transfected cell line studied exhibits modified phenotypic characteristics resulting in the lack of expression of Ang II receptors. However, this seems unlikely since the human renal cell line utilized has been shown to possess most of the receptors of the parental cells [13, 33].

In conclusion, this study demonstrates that the human collecting duct is not a target site for Ang II but for its metabolite, Ang IV. Further studies are needed to examine the physiological consequences of this finding.

Acknowledgments

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