Characterization and Signaling of the AT4 Receptor in Human Proximal Tubule Epithelial (HK-2) Cells

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Abstract. 125I-divalinal-angiotensin IV (metabolically resistant analog of angiotensin IV) was used as a receptor ligand to identify the expression and properties of the angiotensin AT4 receptor in epithelial HK-2 cells (an immortalized cell line derived from adult human proximal tubules). Saturation binding isotherms revealed that HK-2 cells contain a saturable 125I-divalinal-angiotensin IV binding site with an affinity of 3 nmol/L and a density of 508 fmol/mg protein. An analysis of ligand specificity showed that only angiotensin AT1 receptor ligands (angiotensin IV and divalinal-angiotensin IV) competed with both a high- and low-affinity binding site. GTPγS and dithiothreitol did not affect 125I-Ang IV or 125I-divalinal-Ang IV binding, suggesting that the AT4 receptor was not G-protein coupled and did not require sulfhydryl bonds for receptor affinity. Activation of the AT4 receptor caused a complex concentration-dependent rise in [Ca2+]i, an elevation in [Na+]i, and increased mitogen-activated protein kinase activity. These results suggest that human proximal tubule epithelial cells contain functional AT4 receptors that are pharmacologically similar to the AT4 receptor described in more distal segments of the nephron. Furthermore, the AT4 receptor uses several intracellular signaling pathways to convey information.

An angiotensin binding site that demonstrates high specificity and affinity for the hexapeptide angiotensin (3-8) (commonly known as Ang IV) has been described. This novel binding site has been termed the AT4 receptor and is pharmacologically distinct from Ang II type AT1 and AT2 receptors. The AT4 receptor is distributed throughout the body, is present in a vast number of species, and seems to be involved in a number of diverse functions in cognition, cell growth, and cardiovascular regulation. The kidney contains AT4 receptors and can generate Ang IV from Ang II and Ang III by the hydrolytic action of aminopeptidases (1,3) and potentially from Ang I-(3-10) by an angiotensin-converting enzyme–dependent pathway (4). Autoradiographic and radioligand binding studies have identified possible AT4 receptors on cultured rat mesangial cells (5), microvilli and cell bodies of rat proximal convoluted and straight tubules (6), apical and basolateral membranes of rabbit cortical tubules (7), cultured opossum proximal tubule cells (8), cultured human collecting duct cells (9), cultured rabbit collecting duct cells (10), and cultured bovine distal tubule/collection duct cells (11). Activation of the renal AT4-receptor system has been reported to result in increased superficial cortical blood flow (12), inhibition of energy-dependent proximal tubule transport (6), increased plasminogen-activator inhibitor-1 (PAI-1) expression in proximal tubule cells (13), and an increase in intracellular calcium signaling activity in mesangial (5), proximal tubule (8), and distal/collection duct cells (11).

The only study that has pharmacologically characterized the AT4 receptor in proximal tubule cells was performed on cultured opossum OK7A proximal tubule epithelial cells and demonstrated that Ang IV binding to the receptor was inhibited by GTPγS (uncouples G-protein linked receptors) and dithiothreitol (sulfhydryl reducing agent) (8). These findings suggest that the proximal tubule AT4 receptor likely was coupled to a G-protein and required sulfhydryl bonds for high-affinity ligand binding. In contrast, Ang IV binding to the AT4 receptor in cultured human collecting duct cells and cultured bovine distal tubule/collection duct cells was insensitive to GTPγS and dithiothreitol (9,11). These conflicting reports may be resolved by the recent finding of multiple AT4 receptor isoforms (14). Therefore, the possibility exists that the proximal tubule may express an angiotensin AT4 receptor isoform that is distinct from that found in more distal segments of the nephron. To address this question, we examined whether the AT4 receptor was expressed in HK-2 cells (an immortalized epithelial cell line derived from adult human proximal tubules (15)) and then characterized properties of the receptor to determine whether it resembled the AT4 receptor isoform described in opossum proximal tubule cells or more distal renal epithelial cell types.

Materials and Methods

Cell Culture
SV-40 immortalized HK-2 cells possess morphologic and transport characteristics of adult human proximal tubule epithelial cells (15). The cells were grown in an atmosphere of 95% air/5% CO2, at 37°C, in keratinocyte serum-free media supplemented with bovine pituitary extract (30 to 40 μg/ml), epidermal growth factor (7 to 20 ng/ml), penicillin (50 IU/ml), streptomycin (50 μg/ml), and amphotericin B (5
μg/ml). Cultures were refed with fresh media every 2 to 3 d. Radio-
ligand binding experiments were performed on confluent HK-2 cells at
passages 2 to 19 (after thawing frozen cell stocks of unknown
passage) that had been cultured for 2 to 3 wk, whereas intracellular ion
measurements were performed on subconfluent cells that had been
cultured for 5 to 7 d.

Cell Membrane Preparation

Confluent HK-2 cells grown in 75 cm² flasks were washed once
with ice-cold phosphate-buffered saline (PBS) followed by the addi-
tion to the flask of 2 ml of ice-cold isotonic buffer (150 mmol/L NaCl,
50 mmol/L Tris, 50 μmol/L Plummer’s inhibitor [carboxypeptidase
inhibitor], 20 μmol/L bestatin [aminopeptidase inhibitor], 5 mmol/L
thiothreitol (0.1 mol/L ethylenediaminetetraacetate [EDTA], 1.5 mmol/L 1,10-phenanthro-
line [divalent ion chelators], and 0.1% heat-treated bovine serum albumin
at pH 7.4). The cells were dislodged by scraping with a rubber
police man, collected in a centrifuge tube, and homogenized in
10 ml of isotonic buffer for approximately 10 s. The homogenate
was centrifuged at 40,000 × g for 30 min at 4°C. The supernatant
was discarded, the pellet was rehomogenized in 10 ml isotonic buffer,
and the high-speed centrifugation was repeated. The final pellet
was resuspended in isotonic buffer to a working concentration of 1 mg
protein/ml.

Bovine adrenal gland and rat liver were obtained from the local
abattoir and from decapitated adult male Sprague-Dawley rats, re-
spectively. The tissues were immediately homogenized in 10 ml of
hypotonic buffer (50 mmol/L Tris, 1 mmol/L EDTA (pH 7.4) at 4°C)
for approximately 10 s. The homogenate was centrifuged at 5000 × g for 10 min at 4°C, the supernatant was saved on ice, and the
pellet was resuspended in 10 ml of hypotonic buffer, rehomogenized,
and recentrifuged. The supernatants were combined and centrifuged at
40,000 × g for 30 min at 4°C. The resulting bovine adrenal gland
membrane pellet and rat liver membrane pellet were then resuspended
in 10 ml of isotonic buffer and homogenized, and the high-speed
centrifugation was repeated. The final pellets were resuspended
in isotonic buffer to a working concentration of 1 mg protein/ml.

Radioreceptor Assays

HK-2 cell membranes (50 μg) were incubated in a total volume of
250 μl of assay buffer (isotonic buffer). Incubations were performed at
22°C either for 45 min with 0.6 nmol/L [125I]-divalinal-Ang IV or for
30 min with 0.6 mmol/L [125I]-Ang IV, and nonspecific binding was
assessed in the presence of 1 to 10 μmol/L unlabeled divalinal-Ang
IV or Ang IV, respectively. Bound and free radioligands were sepa-
rated by vacuum filtration in a cell harvester using No. 32 glass fiber
filters was measured using a γ counter.

To investigate whether angiotensin receptors were G-protein
linked, we first preincubated the membrane preparations at 22°C for
60 min in the assay buffer supplemented with 5 mmol/L MgCl₂
and 125I- peptide binding was examined in the assay buffer supplemented with increasing concentrations of di-
thiothreitol (0.1 μmol/L to 0.1 mol/L).

Sodium Dodecyl Sulfate-Polyacrylamide Gel
Electrophoresis Analysis of the HK-2 AT₄ Receptor

HK-2 cell membranes and bovine adrenal gland membranes were
generated as described in the Cell Membrane Preparation section.
Membrane proteins were solubilized in buffer containing 1% CHAPS,
50 mmol/L Tris (pH 6.8), and 5 mmol/L EDTA at 4°C for 2 h. The
solubilized AT₄ receptor was then covalently cross linked with [125I-
Tyr-]benzophenone-6-Gly⁷-Ang IV (125I-BP-Ang IV), in the absence or
presence of angiotensin receptor ligands, and subjected to dithiothe-
itol-reduced, sodium dodecyl sulfate-polyacrylamide gel electro-
phoresis (SDS-PAGE) analysis using a previously described method
(16). After electrophoresis, the acrylamide gel was vacuum dried and
exposed to x-ray film for 3 to 4 wk at −70°C.

Measurements of Cytosolic Calcium and Sodium

All studies were performed using a standard bath solution contain-
ing 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl₂, 2 mmol/L
CaCl₂, 10 mmol/L HEPES, and 6 mmol/L glucose (pH 7.4 with
NaOH). Fluorometric determination of cytosolic calcium or sodium
used subconfluent monolayers of HK-2 cells grown on glass covers-
ips. For cytosolic calcium measurements, cells were loaded with 2
μmol/L fura 2-acetoxymethyl ester for 15 min, then washed for 30
min in standard bath solution at room temperature. For cytosolic sodium
measurements, cells were loaded with 5 μmol/L SBFI for 30
min, then washed for 30 min in standard bath solution at room
temperature. Coverslips were then placed in a temperature-regulated
chamber that sat on the stage of an inverted microscope that was
mounted with a micropipette perfusion system. Studies were per-
formed at 37°C on groups of two to eight cells whose fluorescence
was delimited by an adjustable window. Fura 2 or SBFI was alter-
nately excited at wavelengths of 340 nm and 380 nm, and the emission
at 510 nm was measured with a photomultiplier tube using a Photo-
scan-2 fluorescence photometry system (Nikon, Foster City, CA). The
ratio of the 340/380 nm emission intensity was converted to an actual
calcium concentration using a fura 2 calibration table without deter-
mination of minimum and maximum values in individual cells. Con-
sequently, the cytosolic calcium values must be interpreted as approx-
imate concentrations. Only the ratio of the 340/380 nm emission
intensity of SBFI is reported for cytosolic sodium measurements.

Western Blot Analysis of Mitogen-Activated Protein
Kinase Activation

HK-2 cells were grown to confluence in supplemented keratinocyte
medium. Cells were dislodged by gentle trypsinization, washed, and
resuspended in supplemented keratinocyte medium. Aliquots of cell
suspensions were incubated with serial dilutions of angiotensin pep-
tides for 5 min at 37°C, and the reaction was terminated by lysing the
cells with sample-loading buffer (13% 0.5 mol/L Tris-HCl [pH 6.8],
10% glycerol, 2% SDS, 0.01% bromophenol blue, 5% β-mercapto-
ethanol). Proteins within HK-2 cell lysates were resolved by SDS-
PAGE (10% polyacrylamide minigel with a 4% polyacrylamide stack-
gel) and transferred to polyvinylidene difluoride membranes.
The polyvinylidene difluoride transfer membranes were blocked at
room temperature for 1 h with 3% bovine serum albumin in PBS contain-
ing 0.1% Tween-20 (BSA-PBST) and then incubated for 2 h with a
phospho-specific p44 and p42 mitogen-activated protein (MAP) ki-
nase (extracellular signal-regulated kinase-1 [Erk-1] and Erk-2, re-
spectively) primary monoclonal antibody or phospho-specific p38
MAP kinase primary monoclonal antibody (all 1:1000 dilution in
BSA-PBST). These antibodies detect only the phosphorylated (acti-
verted) form of the MAP kinases. The Western blots were then washed
with PBST and incubated for 2 h with an anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (1:75,000 dilution in BSA-PBST). Phosphorylated MAP kinase proteins were detected by incubating PBST-washed blots with a chemiluminescence substrate solution followed by exposure to Kodak X-Omat AR film (Rochester, NY).

Iodination of Peptides

Ang IV, divalinal-Ang IV, and BP-Ang IV were monoiiodinated using a previously described chloramine T method (11).

Materials

We received gifts of HK-2 cells from Dr. Richard A. Zager (Fred Hutchinson Cancer Research Center, Seattle, WA), WSU-1291 (divalinal-Ang IV) and BP-Ang IV from Dr. Joseph W. Harding (Washington State University, Pullman, WA), DuP 753 (losartan) from DuPont/Merck Pharmaceuticals (Wilmington, DE), and PD 123319 from Parke-Davis (Ann Arbor, MI). Angiotensins peptides were purchased from Sigma (St. Louis, MO) and Bachem (Torrance, CA), fura 2-acetoxymethylster and SBF1 from Molecular Probes (Eugene, OR), keratinocyte-serum free media and supplements from Life Technologies (Grand Island, NY), phospho-specific MAP kinase antibodies from New England Biolabs (Beverly, MA), and anti-rabbit IgG: horseradish peroxidase from Transduction Laboratories (Lexington, KY).

Statistical Analyses

All values quoted represent means ± SEM. Results of radioligand binding experiments were analyzed by InPlot4 (GraphPad Software Inc., San Diego, CA). Phosphorylated MAP kinase protein levels were quantified by Sigmascan/Image measurement software (Jandel Scientific, San Rafael, CA). One-way and two-way ANOVA and an appropriate post hoc test were used to analyze multiple groups. Statistical significance was at the 5% level.

Results

Unless otherwise noted, each data point reflects the results from two experiments conducted in duplicate. This minimal number of experiments for each data point was necessary because of the low protein yield from HK-2 cell cultures and the considerable cost of the keratinocyte medium. For example, culturing HK-2 cells in 10 75-cm2 flasks for 2 to 3 wk yielded the considerable cost of the keratinocyte medium. Phosphorylated MAP kinase protein levels were quantified by Sigmascan/Image measurement software (Jandel Scientific, San Rafael, CA). One-way and two-way ANOVA and an appropriate post hoc test were used to analyze multiple groups. Statistical significance was at the 5% level.

Radioligand Binding Study

Figure 1 illustrates the comparative binding of 125I-divalinal-Ang IV (AT4 receptor ligand) and 125I-sar1,ile8 Ang II (AT1/AT2 receptor ligand) to HK-2 cell membranes as a function of protein concentration. 125I-divalinal-Ang IV displayed high specific binding to HK-2 cell membranes, suggesting the possible presence of AT4 receptors, whereas 125I-sar1,ile8 Ang II bound negligibly and likely reflected the absence or low amount of AT1 and/or AT2 receptors in this renal epithelial cell line. Specific binding of 125I-divalinal-Ang IV to HK-2 membranes increased as a function of time, and apparent steady state was reached at approximately 20 min and remained stable for at least 100 min thereafter (Figure 2A). The well-maintained steady state conditions suggested minimal metabolism of the radiolabeled ligand during the period of incubation. This was confirmed by metabolism studies that demonstrated that 125I-divalinal-Ang IV was 98% and 97% intact at 45 min and 120 min of incubation, respectively (not shown). On the basis of the above results, all further experiments used a protein concentration of 50 μg/tube that lay in the linear portion of the protein concentration-binding response curve and an incubation time period of 45 min that was within the plateau phase of the association curve. Under these radioreceptor binding conditions, the amount of nonspecific 125I-divalinal-Ang IV bound to the tissue was ≤20% of total binding. Specific binding of 125I-divalinal-Ang IV was rapidly reversible after the addition of 1 μmol/L unlabeled divalinal-Ang IV to steady state conditions (Figure 2B). Calculation of the Kd from association and dissociation rate constants indicated that 125I-divalinal-Ang IV bound with high affinity to the membrane receptor with a kinetic Kd of 3.2 nmol/L. The results from nonlinear regression analysis of saturation binding isotherms are shown in Figure 3 and demonstrate that 125I-divalinal-Ang IV bound to HK-2 cells with high-affinity and density (apparent Kd = 3.0 ± 0.2 nmol/L; Bmax = 508 ± 30 fmol/mg protein, n = 3).

To determine whether binding sites with differing affinities for divalinal-Ang IV existed in HK-2 cells, we generated competition curves by incubating 125I-divalinal-Ang IV with increasing concentrations of unlabeled divalinal-Ang IV (10 pmol/L to 10 μmol/L). As shown in Figure 4A, competition data best fit a two-site binding model (P < 0.01) with divalinal-Ang IV competing for 18% of the specific binding with an IC50 value of 0.13 nmol/L and competed with the remaining 82% of binding sites with an IC50 value of 128 nmol/L. Pharmacologic characterization of the binding sites indicated that the AT4 receptor ligand Ang IV competed for both binding sites (P < 0.001; 14% and 86% of total binding with an IC50 value of 0.08 nmol/L and 76 nmol/L, respectively), whereas AT1 receptor ligands (losartan and Ang II), AT2 receptor ligands (PD 123319 and Ang II), and Ang-(1-7) receptor
ligands [Ang-(1-7) and D-Ala⁷ Ang-(1-7)] did not compete with either binding site (Figure 4B).

Preincubation of HK-2 cells with GTPγS (uncouples G-protein linked receptors) did not affect ¹²⁵I-divalinal-Ang IV or ¹²⁵I-Ang IV binding to the membrane AT₄ receptor yet decreased more than 90% of ¹²⁵I-Ang II binding to angiotensin AT₁ receptors (predominant subtype) in rat liver membranes (Figure 5A). Binding of ¹²⁵I-divalinal-Ang IV or ¹²⁵I-Ang IV to HK-2 cells was also insensitive to the presence of the sulphydryl reducing agent dithiothreitol at concentrations that abolished ¹²⁵I-Ang II binding to rat liver angiotensin AT₁ receptors (Figure 5B). These results suggested that the radioligand-bound receptor protein studied in HK-2 cell membranes was distinct from the AT₁ receptor and that the binding site of the AT₄ receptor did not require G-protein or sulphydryl bonds for binding affinity.

**SDS-PAGE Study**

To ascertain whether the α-subunit (containing the binding site) of the HK-2 AT₄ receptor protein was similar to that described in other cell types (14,16–18), we covalently labeled the solubilized bovine adrenal and HK-2 AT₄ receptor with ¹²⁵I-BP-Ang IV and analyzed it by dithiothreitol-reduced SDS-PAGE. As shown in Figure 6A, autoradiography of the bovine adrenal polyacrylamide gel revealed a single molecular weight band of approximately 165 kD for the untreated bovine adrenal

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**Figure 2.** Time course for the association (A) and dissociation (B) of ¹²⁵I-divalinal-Ang IV to and from HK-2 cell membranes, respectively. Data represent the results from one of two experiments.

**Figure 3.** Saturation binding isotherm for ¹²⁵I-divalinal-Ang IV in HK-2 cell membranes. Data represent the results from one of three experiments.

**Figure 4.** Competition for specific ¹²⁵I-divalinal-Ang IV binding to HK-2 cell membranes by divalinal-Ang IV (A; n = 6). Nonspecific binding was measured using 10 μmol/L divalinal-Ang IV and subtracted from each point. (B) The competition curves generated by the competition of total ¹²⁵I-divalinal-Ang IV binding to HK-2 cell membranes by Ang IV (n = 5) and other angiotensin receptor ligands (n = 2, each). For some data points, the SEM bar was less than the symbol size of the mean value.

**Figure 5.** Time course for the association (A) and dissociation (B) of ¹²⁵I-divalinal-Ang IV to and from HK-2 cell membranes, respectively. Data represent the results from one of two experiments.

**Figure 6.** Autoradiography of the bovine adrenal polyacrylamide gel revealed a single molecular weight band of approximately 165 kD for the untreated bovine adrenal.
AT$_4$ receptor (control, lane 4). This value is in agreement with previous estimations of the molecular weight of the $\alpha$-subunit of the bovine adrenal AT$_4$ receptor (14,16). The labeled protein band was present after pretreatment of the receptor with 1 $\mu$mol/L losartan and PD123319 (lane 3) but absent after 1 $\mu$mol/L divalinal-Ang IV (lane 2) or 1 $\mu$mol/L Ang IV (lane 1) pretreatment. Figure 6B demonstrates that 125I-BP-Ang IV labeled a protein of similar molecular size as the bovine adrenal AT$_4$ receptor (lane 5) in untreated solubilized HK-2 receptors (control, lane 1) and in HK-2 receptors that were pretreated with either 1 $\mu$mol/L losartan (lane 3) or 1 $\mu$mol/L PD 123319 (lane 4). However, the labeled protein band was absent in HK-2 receptors that were pretreated with 1 $\mu$mol/L Ang IV (lane 2).

**Intracellular Calcium Study**

HK-2 cells were exposed to a concentration of Ang IV (AT$_4$ receptor agonist) or divalinal-Ang IV (putative AT$_4$ receptor antagonist) that was varied from 0.1 nmol/L to 1 $\mu$mol/L. As shown in Figure 7, both Ang IV and divalinal-Ang IV were capable of producing a rise in $[\text{Ca}^{2+}]_i$. Although the concentration-response curves for Ang IV and divalinal-Ang IV were similar between 0.1 and 10 nmol/L, they diverged at the higher concentrations ($\geq$100 nmol/L) with Ang IV eliciting a diminished $[\text{Ca}^{2+}]_i$ response compared with that observed with divalinal-Ang IV at 1 $\mu$mol/L concentrations ($P < 0.05$; two-way ANOVA with exclusion of $10^{-7}$ M Ang IV data; Figure 7D). The absence of a detectable $[\text{Ca}^{2+}]_i$ response to 0.1 and 1 $\mu$mol/L Ang IV was not due to desensitization, because it occurred even when the highest concentration of Ang IV was administered first followed by lower concentrations that produced greater $[\text{Ca}^{2+}]_i$ responses (not shown) or interspersed between repeated 10 nmol/L concentrations that produced maximal increases in $[\text{Ca}^{2+}]_i$ (as shown in Figure 7A). The $[\text{Ca}^{2+}]_i$ response to 10 nmol/L Ang IV was not significantly altered by a 3-min pretreatment of cells with 1 $\mu$mol/L losartan (not shown). Exposing cells to 10 nmol/L Ang IV for longer periods (approximately 5 min) resulted in an initial transient increase in $[\text{Ca}^{2+}]_i$ that fell to a plateau level and remained constant until the exposure to Ang IV was terminated (far right response shown in Figure 7A). The $[\text{Ca}^{2+}]_i$ response to divalinal-Ang IV in HK-2 cells was complex and variable. In general, we found that short exposure (approximately 20 s) of HK-2 cells to divalinal-Ang IV either produced a transient

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**Figure 5.** Effect of increasing concentrations of GTP$\gamma$S (A) and dithiothreitol (B) on 125I-Ang IV and 125I-divalinal-Ang IV binding to HK-2 cell membranes ($n = 2$, each) and 125I-Ang II binding to rat liver membranes ($n = 3$). For some data points, the SEM bar was less than the symbol size of the mean value.

**Figure 6.** Reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the ability of angiotensin receptor ligands to block the covalent binding of 125I-BP-Ang IV to AT$_4$ receptors in bovine adrenal tissue and HK-2 cells. Compounds (each 1 $\mu$mol/L) used to treat bovine adrenal tissue (A) were as follows: lane 1, Ang IV (AT$_4$ receptor agonist); lane 2, divalinal-Ang IV (putative AT$_4$ receptor antagonist); lane 3, losartan and PD 123319 (AT$_1$ and AT$_2$ receptor antagonist, respectively); lane 4, no addition of compounds (total binding). Compounds (each 1 $\mu$mol/L) used to treat HK-2 cells (B) were as follows: lane 1, no addition of compounds (total binding); lane 2, Ang IV; lane 3, losartan; lane 4, PD 123319; lane 5, total binding to bovine adrenal tissue (radioactive counts were not adequately diluted to match those present in the HK-2 control sample, which resulted in a large, dense image of the 125I-BP-Ang IV labeled bovine adrenal protein band).
concentration-dependent rise in \([\text{Ca}^{2+}]_i\) that slowly recovered toward basal levels or resulted in a concentration-dependent elevated and maintained rise in \([\text{Ca}^{2+}]_i\). However, long-term exposure of cells to divalinal-Ang IV (at concentrations >1 nmol/L for more than 1 min) consistently resulted in a concentration-dependent elevation in \([\text{Ca}^{2+}]_i\), that was maintained after the termination of the divalinal-Ang IV perfusion (Figure 7B). This phenomenon was most apparent with cells that were exposed to 1 \(\mu\text{mol/L}\) divalinal-Ang IV producing an immediate rise in \([\text{Ca}^{2+}]_i\), that tended to wane before once again increasing in magnitude despite terminating the divalinal-Ang IV perfusion (Figure 7B). The photomultiplier tube averages the calcium response from the group of cells under investigation. Therefore, it was unclear whether all cells or subgroups of cells responded to 1 \(\mu\text{mol/L}\) divalinal-Ang IV in this manner. Consequently, we used digital imaging fluorescence microscopy, which allowed simultaneous measurements of \([\text{Ca}^{2+}]_i\) from individual cells within groups of cells. These experiments could be performed only at room temperature and revealed that all cells responded to divalinal-Ang IV with a rise in \([\text{Ca}^{2+}]_i\). However, there seemed to be subgroups of cells that responded to divalinal-Ang IV with either a short or long latency of onset (Figure 7C). Calculation of the group response, by averaging the individual cell calcium responses, produced a curve that was similar in shape to that observed with the photomultiplier tube calcium imaging system.

**Intracellular Sodium Study**

As shown in Figure 8, continuous perfusion of HK-2 cells with 10 nmol/L Ang IV resulted in a slow increase in the fluorescence (340/380 nm) ratio that had a long latency for onset. In contrast, perfusion of cells with either 10 nmol/L divalinal-Ang IV or 5 mmol/L ouabain (\(\text{Na}^+\)-\(\text{K}^+\)-ATPase inhibitor) resulted in a rapid increase in the fluorescence ratio. An increase in the fluorescence ratio indicates a rise in \([\text{Na}^+]_i\).

**MAP Kinase Signaling Study**

HK-2 cells had low basal phosphorylated Erk-2 protein levels. All concentrations of Ang IV and divalinal-Ang IV caused an increase in the phosphorylated levels of Erk-2 above control values \((P < 0.05, \text{one-way ANOVA with repeated measures})\), indicating an increased catalytically active form of this signaling protein (top and middle panels of Figure 9A, respectively; cumulated response shown in Figure 9B). Basal Erk-1 activity and its stimulation by both AT4 receptor ligands...
was apparent only after long-term exposure of the protein-bound membrane to x-ray film (not shown). Basal phosphorylated p38 kinase activity could not be detected in HK-2 cells irrespective of the time of exposure to x-ray film. We examined only the effect of Ang IV on p38 kinase activity and found that the peptide could dramatically increase p38 kinase activity (bottom panel of Figure 9A).

Discussion

The results of the present study demonstrate the presence of AT₄ receptors in HK-2 cells, a line derived from adult human proximal tubule epithelial cells. This cell line demonstrates morphologic and biochemical markers that are characteristic of proximal tubule epithelia (15). The predominant Ang receptor expressed in HK-2 cells was shown to be the AT₄ receptor, which was of high affinity (apparent and kinetic Kₐ values were approximately 3 nmol/L) and density (508 fmol/mg protein). ¹²⁵I-divalinal-Ang IV binding to HK-2 cell membranes decreased concentration-dependently by both divalinal-Ang IV and Ang IV with the best-fit curve of the competition data indicating that the peptides bound to both a high-affinity site (approximately 18% of binding sites) and low-affinity site (approximately 82% of binding sites). A similar distribution of high- and low-affinity binding sites for ¹²⁵I-Ang IV has also been reported in opossum proximal tubule epithelial (OK7A) cells (8). ¹²⁵I-Ang IV binding to opossum OK7A cells was inhibited by GTPγS and dithiothreitol, suggesting that the proximal tubule epithelial receptor was G-protein coupled and required intact sulfhydryl bonds for high-affinity binding. In contrast, the results of the present study suggest that the AT₄ receptor present in human proximal tubular epithelial cells share similar pharmacologic and coupling characteristics as the AT₄ receptor described in more distal segments of the nephron (9,11) and in nonrenal tissues (2,17), e.g., receptor was unlikely to be coupled to a “classic” G-protein, did not require sulfhydryl bonds for binding affinity, and had low affinity for known ligands of AT₁, AT₂ and Ang-(1-7) receptors. We also found that reduced SDS-PAGE analysis of the photoaffinity-labeled α-subunit of the HK-2 AT₄ receptor produced a single molecular band that was of similar size as the α-subunit of the AT₄ receptor found in bovine adrenal membranes, suggesting that the binding subunit of the AT₄ receptor in human proximal tubule cells was similar to the most common AT₄ receptor isoform encountered in peripheral tissues (14). Therefore, our findings do not support the notion that the proximal tubule contains an unusual AT₄ receptor with properties that are distinct from the AT₄ receptor found in more distal segments of the nephron.

We examined whether the AT₄ receptor identified in human proximal tubule epithelial cells was biologically active by examining the intracellular signaling mechanism(s) associated with AT₄ receptor activation. Investigators have previously shown that stimulation of the AT₄ receptor system can elevate intracellular Ca²⁺ levels (5,8,11), cGMP production (19), and nitric oxide biosynthesis (12,19,20), whereas others have reported minimal or no influence on classic second messenger systems such as cAMP, cGMP, nitric oxide, Ca²⁺, inositol phosphates, and arachidonic acid (8,9,18). Our results suggest that a rise in [Ca²⁺]ᵢ is one mechanism by which ligand binding to the HK-2 AT₄ receptor is transduced into an intracellular signal. We found that Ang IV (AT₄ receptor agonist) elicited an increase in [Ca²⁺]ᵢ only at low nanomolar concentrations. The reasons for a diminished [Ca²⁺]ᵢ response to high concentrations of Ang IV (≥100 nmol/L) are unclear but do not seem to be due to desensitization of the AT₄ receptor. In contrast, divalinal-Ang IV (putative AT₄ receptor antagonist) produced only a concentration-dependent increase in [Ca²⁺]ᵢ that was complex in nature. Cells were continuously perfused with vehicle or peptide solutions, thereby preventing differences in peptide metabolism stability (or the local build-up of metabolites) influencing the cellular biologic response. We recently reported a similar biphasic and monophasic concentration-dependent action of Ang IV and divalinal-Ang IV on [Ca²⁺]ᵢ, respectively, in bovine kidney epithelial cells and speculated on the possible role of a low-affinity AT₄ receptor or non-AT₄ receptor accounting for the diminished [Ca²⁺]ᵢ response to high concentrations of Ang IV (11). Further studies clearly are needed to address the mechanism(s) responsible for
the concentration-dependent biphasic \([\text{Ca}^{2+}]_i\) response to Ang IV in renal epithelial tissue, as well as its physiologic significance in cell function.

Activation of the AT\(_4\) receptor system in HK-2 cells altered intracellular Na\(^+\) levels. Perfusion of cells with Ang IV caused a slow increase in \([\text{Na}^+]_i\), which was greater than that observed with vehicle infusion, whereas both divalinal-Ang IV and ouabain (Na\(^+\)-K\(^+\)-ATPase inhibitor) produced rapid increases in \([\text{Na}^+]_i\). Our results cannot distinguish whether the AT\(_4\) receptor ligand-induced rise in \([\text{Na}^+]_i\) was due to an increase in Na\(^+\) influx into the cell and/or a decrease in Na\(^+\) efflux from the cell. Nevertheless, we speculate that a decrease in the activity of Na\(^+\) influx pathways most likely contributed to the Ang IV-mediated rise in \([\text{Na}^+]_i\), because (1) Ang IV acts via the AT\(_4\) receptor to inhibit ouabain-suppressible oxygen consumption in nystatin-treated (bypasses the rate limiting step of apical Na\(^+\) influx into the cell) rat proximal tubules, presumably reflecting a decrease in Na\(^+\)-K\(^+\)-ATPase activity (6), and (2) Ang IV infused directly into the left renal artery of pentobarbital-anesthetized male Wistar rats with surgically and chemically denervated left kidneys, at concentrations that do not seem to activate AT\(_1\) receptors (1 nmol/min per kg for 30 min), resulted in a marked elevation in left kidney urinary Na\(^+\) and water excretion (341 ± 97% and 61 ± 9%, respectively; \(P < 0.05\) each), with no change in urinary K\(^+\) excretion, total renal blood flow, or renal vascular resistance (unpublished results, \(n = 4\)). Consistent with the notion that the renal AT\(_4\) receptor system could potentially be involved in regulating kidney hydroelectrolyte transport is the recent report that kidney AT\(_4\) receptor expression was increased by high dietary Na\(^+\) intake in Wistar Kyoto rats that remain normotensive (21). In contrast to the slow kinetics of the Ang IV response, divalinal-Ang IV caused a rapid and robust increase in \([\text{Na}^+]_i\), that was similar to that observed with ouabain, a Na\(^+\)-K\(^+\)-ATPase inhibitor. It is unclear which factor(s) contributes to the rapid kinetics and/or magnitude of the divalinal-Ang IV response (both \([\text{Na}^+]_i\), and \([\text{Ca}^{2+}]_i\) compared with Ang IV. However, we believe that the reason(s) is unlikely to be related to known differences in their metabolic stability (3,22) because HK-2 cells were continuously perfused with peptide and/or a difference in peptide affinity for the AT\(_4\) receptor (see Figure 4).

Adding to the complexity of the stimulatory action of divalinal-Ang IV on \([\text{Na}^+]_i\), we previously reported that active Na\(^+\) transport in rat proximal tubules (as measured by nystatin-stimulated, ouabain-suppressible tissue oxygen consumption rates) was not significantly altered after acute (≤2 min) or chronic (≥10 min) incubation with divalinal-Ang IV (6). In fact, the oxygen consumption response of nystatin-stimulated rat proximal tubules to an acute challenge with divalinal-Ang IV was highly variable; 42% of the responses (8 of 19 data points) reflected some degree of AT\(_4\) receptor agonist activity. We and others have also reported that divalinal-Ang IV can block the functional response of Ang IV in several physiologic systems without manifesting significant intrinsic AT\(_4\) receptor activity (6,12,22). However, our more recent studies (11,23, present study) and unpublished work suggest that divalinal-Ang IV possesses properties that can be both similar and distinct from Ang IV and that divalinal-Ang IV’s physiologic activity (AT\(_4\) receptor antagonism or agonism) may be related to its concentration, the physiologic process, and/or cell type that is being examined. Our findings should not be interpreted as suggesting that divalinal-Ang IV can be used as an AT\(_4\) receptor agonist in some biologic systems but rather that the biologic properties of divalinal-Ang IV are complex and that it cannot be regarded as simply an AT\(_4\) receptor antagonist with no intrinsic biologic activity in all circumstances.

Members of the MAP kinase family are structurally related protein kinases that act to link extracellular signals to an integrated series of regulated cellular events. They include Erk, which generally are activated by growth factors and are particularly important for cell division, growth, and differentiation, and p38 kinases and Jun N-terminal kinases/stress-activated protein kinases, which are activated by cellular stresses (e.g., exposure to inflammatory cytokines, endotoxins, heat shock, osmolar stress) and are involved in inflammation and apoptosis (24). Angiotensin peptides such as Ang II and Ang-(1-7) are known to influence cell biologic activity by regulating MAP kinase activity (24,25). Therefore, we reasoned that the Ang IV-AT\(_4\) receptor system may also use the MAP kinase signaling pathway to convey information within the cell, especially because the AT\(_4\) receptor system has been reported to regulate cell growth (26). Our results demonstrate that HK-2 cells had low basal protein levels of phosphorylated Erk-1 and Erk-2 with no detectable basal expression of the phosphorylated p38 kinase. Both Ang IV and divalinal-Ang IV produced an increase in the phosphorylation (activation) of Erk-1, Erk-2, and p38 kinase. A recent study reported that HK-2 cells that were exposed to Ang IV had no effect on cell proliferation but did increase PAI-1 mRNA expression (13). PAI-1 is an enzyme involved in regulating (inhibitory) plasminogen activators that are responsible for converting plasminogen to the active enzyme plasmin. Plasmin can degrade fibrin and activate matrix metalloproteases that are mainly involved in the degradation of the extracellular matrix (27). Several investigators showed that pharmacologic blockade of the MAP kinase pathway suppresses PAI-1 biosynthesis in cultured cells (28,29). Therefore, the reported ability of Ang IV to increase PAI-1 expression in HK-2 cells (13) may be due to the HK-2 AT\(_4\) receptor being coupled to the intracellular MAP kinase signaling pathway (present study). However, we emphasize that the activity of the AT\(_4\) receptor system on MAP kinase activity may be cell specific as both Ang IV and divalinal-Ang IV caused a concentration-dependent attenuation of phosphorylated Erk-2 protein expression in Mardin-Darby bovine kidney epithelial cells, which possess distal tubule and collecting duct characteristics (23).

The results from intracellular ion and signaling studies provide evidence for a functional AT\(_4\) receptor system in HK-2 cells. This conclusion is supported by the recent report that Ang II and Ang IV stimulated PAI-1 expression in HK-2 cells and that Ang II’s effect was mediated exclusively by the formation of Ang IV acting on a non-AT\(_1\), non-AT\(_2\) receptor (13). It is likely that the AT\(_4\) receptor mediated Ang IV’s effect
on PAI-1 mRNA expression because it is the predominant Ang receptor expressed in HK-2 cells (present study). Gesualdo et al. (13) also reported that HK-2 cells responded to Ang II with an increase in Transforming growth factor-β mRNA expression that could be abolished by pretreating cells with losartan, an AT1 receptor antagonist. Therefore, the lack of 125I-sar 1 , ile8 Ang II (mixed AT1/AT2 receptor antagonist) binding to HK-2 cell membranes may reflect very low expression of AT1 receptors (and perhaps AT2 receptors) rather than the absence of these Ang receptor subtypes. In which case, it could be argued that the biologic actions of Ang IV and divalinal-Ang IV in HK-2 cells may be mediated by the AT1 subtype of the Ang II receptor. We believe that this is most unlikely because (1) the [Ca2+]i response to Ang IV was unaltered by losartan pretreatment, (2) the slow kinetics of the [Na+]i response to Ang IV is inconsistent with the rapid rise in [Na+]i, (3) <1 s described for Ang II in proximal tubule cells and believed to be mediated by the AT1 receptor (30), (3) submicromolar concentrations of Ang IV seem to have little influence on AT1 receptor-mediated processes (13,31), and (4) both Ang IV and divalinal-Ang IV elicited similar agonist responses, with the latter peptide having no demonstrable affinity for the AT1 receptor (11) and lacking biologic activities associated with known AT1 receptor-mediated events such as a decrease in renal and cerebral blood flow (12,22).

In conclusion, we identified the abundant expression of biologically functional AT4 receptors in HK-2 cells, a cell line that is representative of adult human proximal tubule epithelia. The human proximal tubule epithelial AT4 receptor was shown to have similar pharmacologic and coupling properties as that described in more distal segments of the nephrone and to be linked to the activation of several intracellular signaling and ion regulating systems.

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