Angiotensin (AT$_{1A}$) receptor-mediated increases in transcellular sodium transport in proximal tubule cells

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Angiotensin (AT$_{1A}$) receptors are abundant in proximal tubule epithelial cells (PTEC). Angiotensin II (ANG II) is important in regulating proximal tubule salt and water balance. AT$_{1A}$ receptors are present on apical (AP) and basolateral (BL) surfaces of proximal tubule epithelial cells (PTEC). The molecular mechanism of AT$_{1A}$ function in epithelial tissue is not well understood, because specific binding of ANG II to intact PTEC has not been found and because a number of isoforms of AT receptors are present in vivo. To overcome this problem, we developed a cell line from opossum kidney (OK) proximal tubule cells, which stably express AT$_{1A}$ receptors. Characterization of nontransfected OK cells revealed no evidence of AT$_{1A}$ mRNA (reverse transcriptase-polymerase chain reaction analysis) or protein ($^{125}$I-labeled ANG II binding studies) expression. In cells stably expressing AT$_{1A}$, ANG II binding was saturable, reversible, and regulated by G proteins. Transfected receptors were coupled to increases in intracellular calcium and inhibition of cAMP. The polarity of AT$_{1A}$ expression and function in proximal tubules, transfected cells were grown to confluence on membrane inserts under conditions that allowed selective access to AP or BL surfaces. AT$_{1A}$ were expressed on both AP (K$_d$ = 8.7 nM, B$_{max}$ = 3.33 pmol/mg protein) and BL (K$_d$ = 10.1 nM, B$_{max}$ = 5.50 pmol/mg protein) surfaces. Both AP and BL AT$_{1A}$ receptors underwent agonist-dependent endocytosis (AP receptor: t$_{1/2}$ = 7.9 min, Y$_{max}$ = 78.5%; BL receptor: t$_{1/2}$ = 2.1 min, Y$_{max}$ = 86.3%). In cells transfected with AT$_{1A}$, ANG II caused time- and concentration-dependent increases in transepithelial $^{22}$Na transport (2-fold over control at 20 min) by increasing Na/H exchange. In conclusion, we have established a stable proximal tubule cell line that expresses AT$_{1A}$ on both AP and BL surfaces, undergoes agonist-dependent receptor endocytosis, and is functional, as evidenced by inhibition of cAMP and increases in cytosolic calcium mobilization and transepithelial sodium movement. This cell line should prove useful for understanding the molecular and biochemical regulation of AT$_{1A}$ expression and function in PTEC.

ANGIOTENSIN II (ANG II) actions are mediated by specific receptors on plasma membranes of cells (22). Pharmacological studies and cDNA cloning have identified multiple ANG II receptors, but most known ANG II actions in kidney are associated with the AT$_{1}$ receptor type (25). ANG II is important in the regulation of total body Na. Increases in total body Na as a consequence of renal salt retention occur in clinical states associated with activation of the renin-angiotensin system. ANG II regulates renal Na reabsorption through both indirect and direct pathways. Indirect pathways include ANG II-mediated regulation of aldosterone production, as well as increases in systemic and renal vascular resistance. In addition, ANG II directly regulates proximal tubule Na reabsorption. Using micropuncture and microperfusion techniques, a number of investigators have reported that low concentrations of ANG II activate proximal tubule Na/H exchange and high concentrations of ANG II inhibit Na reabsorption (14, 39, 43, 53).

Despite the clinical observation that high ANG II states are associated with enhanced proximal tubule Na reabsorption, reports on the direct effect of ANG II on proximal tubule Na transport and on the signaling mechanisms that transduce ANG II action have been highly variable. Most studies have used ANG II-dependent regulation of proximal tubule transporters as surrogate indicators of transcellular Na reabsorption. ANG II-dependent increases and decreases in Na/H exchange (11, 27, 32, 39, 53) and Na-K-ATPase (8, 19, 23) have been reported. The surrogate indicators of Na transport, however, may not reflect the effect of ANG II on transcellular Na movement because of the possibility that the signaling enzymes, which transduce ANG II action, could have divergent effects on transport proteins that regulate transcellular Na transport. For example, protein kinase C (PKC) has been reported to activate or inactivate Na/H exchange (26, 36, 51, 52) and to stimulate or inhibit Na-K-ATPase (3, 6, 7, 37).

Which signaling enzymes couple ANG II to Na transport is not clear. We reported that ANG II-dependent increases in Na transport were associated with increases in phospholipase C (PLC) activity and were blocked with a PLC inhibitor (42). PKC dependency has been confirmed by some (12, 34), but others reported no role (15) or a biphasic role (27) of PKC in ANG II regulation of Na transport. As with PLC and PKC, some, but not other, studies suggest that ANG II-dependent inhibition of adenylate cyclase and decreases in cAMP mediate ANG II-dependent increases in Na transport (15, 33, 42). The major difficulty in determining the cellular effects on ANG II on Na reabsorption has been that cell culture systems utilized to study ANG II action express low levels of AT$_{1A}$ receptors. Although membrane preparations of isolated proximal tubules express high levels of ANG II receptors (400–900 fmol/mg protein) (9, 10), ANG II binding data has not been reported in primary cultures of rat or rabbit proximal tubule cells in culture. These observations are further complicated by the possibility that, in vivo, multiple AT receptor subtypes with different...
affinity for agonist and cellular responses could be present. To overcome these potential confounding variables, rabbit AT1A receptor cDNA has been transfected in LLC-PK cells (5). However, transfected cells expressed low levels of AT1A receptors [apical (AP), \( B_{\text{max}} = 7.5 \text{ fmol/mg protein} \); basolateral (BL), \( B_{\text{max}} = 16.6 \text{ fmol/mg protein} \)]. These low levels of binding may not be sufficient to determine the direct effects of AT1A receptors on proximal tubule transporters or on Na reabsorption.

The purpose of this study, therefore, was to develop a proximal tubule cell line stably expressing sufficient numbers of AT1A receptors to determine the direct effects of AT1A receptors, without the interference of other receptor subtypes, on proximal tubule transcellular Na reabsorption. To accomplish this goal, we stably expressed AT1A receptors in an opossum kidney (OK) proximal tubule cell line, which normally does not express AT1A receptors in culture. The results indicate that transfected cells express functional AT1A receptors and that ANG II increases receptor-mediated Na reabsorption at each concentration tested.

MATERIALS AND METHODS

Materials. OK cells were obtained from Dr. John Raymond of the Medical University of South Carolina. Dulbecco’s modified Eagle’s medium nutrient mixture Ham’s F-12 (DME/F-12), trypsin, Genetin, bovine calf serum, and antibiotics were obtained from Life Technologies (Gaithersburg, MD). Tissue culture dishes, flasks, plasticware, and Transwell cell culture inserts were purchased from Costar (Cambridge, MA). The radioiodinated ANG II was purchased from NEN (Boston, MA). Fura 2-AM was from Calbiochem (La Jolla, CA). ANG II was from Sigma Chemical (St. Louis, MO). Losartan and EXP-3174 were generous gifts from Merck (Willington, DE). Guanylinidodiphosphate (GMP-PNP) was from Boehringer-Mannheim (Indianapolis, IN). The cAMP assay kit was from Amersham Life Sciences (Arlington Heights, IL). Taq polymerase and PCR reagents were obtained from Perkin-Elmer (Norwalk, CT). All other molecular biology grade chemicals were obtained from either United States Biochemical (Cleveland, OH) or Fisher Scientific (Pittsburgh, PA).

Stable expression of rat AT1A receptor in OK epithelial cells. The cloning and isolation of a genomic clone for the rat AT1A receptor (M.12), subcloning of the full-length receptor (coding for 359 amino acids) into pBluescript II (pB2/AT1A), and the subsequent incorporation of the receptor into the prcCMV eukaryotic expression vector (pRc2A/AT1A) have been previously described (48). Cells were grown in monolayers in a humidified incubator at 37°C with 5% CO2. After 48 h, the medium was aspirated and replaced with fresh complete medium containing 600 µg/ml Genetin (G418). Every 3rd day, old medium was replaced by fresh selection medium. After 18–21 days of transfection, individual neomycin-resistant colonies were isolated for propagation and analysis. Initially, colonies were screened for \(^{125}\text{I}\text{-labeled ANG II binding. One of the cell lines (T35OK/AT1A)} \) expressing high levels of AT1A receptors was selected for the present study. Passages 6–76 were used for study. AT1A receptor expression has been stable through these passages.

Reverse transcriptase-polymerase chain reaction. To confirm the authenticity of the stable expression of the AT1A receptor and the absence of the receptor transcript in the parental OK cell line, we performed reverse transcriptase-polymerase chain reaction (RT-PCR) analysis on mRNA isolated from OK and T35OK/AT1A cells. Total RNA was prepared from the acid guanidinium thiocyanate-phenol-chloroform method, as previously described (50). Two micrograms of total RNA were annealed to 10 pmol of random hexamer oligonucleotide, and first-strand cDNA was synthesized using 20 units of SuperScript II (Life Technologies) reverse transcriptase. The cDNA of 257 bp corresponding to the coding sequence was then amplified using a sense primer (5′-GTGGCCAAAGCTACCTGCATC3′) and an antisense primer (5′-TGAAATTTCAAAACCTCCTT3′) crossed across all known AT1A sequences in a reaction volume of 100 µl containing 2.5 units of Thermus aquaticus (Taq) polymerase. The cDNA was subjected to 24 cycles of repeated denaturing (30 s at 95°C), annealing (30 s at 50°C), and extension (30 s at 72°C) in a DNA thermal cycler (Perkin-Elmer GeneAmp PCR system 9600). The amplified product was separated on a 1% agarose gel, and the ethidium bromide-stained DNA band was visualized by autoradiography (2, 48).

ANG II receptor binding and internalization studies. Receptor transfected and nontransfected OK cells were grown to semiconfluence on 24-well tissue culture plates for total cell surface \(^{125}\text{I}\text{-ANG II binding studies or were grown to confluency on 6.5-mm diameter, 0.4-µm pore permeable Transwell membranes} \) for differential binding to apical (AP) or basolateral (BL) surfaces (40). Confluence was confirmed by the development of a transepithelial resistance and barrier to transport of \(^{3}\text{H}\text{inulin} \) (40). Assay buffer consisted of 50 mM Tris, pH 7.4, 100 mM NaCl, 5 mM MgCl2, 0.25% bovine serum albumin, and 0.5 mg/ml bacitracin. Binding assays were performed in triplicate. For each experiment, cells were washed twice with ice-cold PBS, and 30 µl of either binding buffer or unlabeled ANG II were added to each well, followed by 270 µl of binding buffer containing 0.04–0.06 nM \(^{125}\text{I}\text{-ANG II} \). To determine differences between AP and BL membrane binding, \(^{125}\text{I}\text{-ANG II} \) was added to either AP or BL buffers. Equilibrium binding was performed for 30 min at 22°C for total cell surface binding and for 180 min at 4°C for AP or BL surface binding. Incubations were terminated by rapid removal of incubation solutions and addition of ice-cold PBS. Cells in plates were dissolved in 200 µl of 0.2 N NaOH. Solutions were transferred to 12 × 75-mm disposable tubes, or inserts were placed directly into tubes, and radioactivity was determined using a Packard Auto-Gamma counter. Results are expressed as specific binding. Specific binding was defined as total binding minus nonspecific binding (in the presence of unlabeled ANG II, 1 µM). Nonspecific binding was <5% of total binding. Values were normalized to the amount of protein determined using Bio-Rad DC protein assay. Results were analyzed using the computer-software GraphPad.
Na₂HPO₄, 4 mM MgCl₂, 0.25% bovine serum albumin, and buffer containing 50 mM Tris, pH 7.4, 100 mM NaCl, 6 mM 24-well plates in DME/F12 supplemented with 10% bovine serum albumin in a 37°C incubator in the presence of 5% CO₂ for 30 min. Cells were moved, and inserts were placed in the transport chamber. 22Na (1 µCi/ml) was added to the AP chamber as transport buffer, which contained (in mM) 128 NaCl, 5 NaHCO₃, 5 KCl, 4 Na₂HPO₄, 1 MgSO₄, 5.0 urea, 5.4 glucose, 2.2 mg/ml bacitracin and exposed to different compounds for 7 min in the same buffer (250 µl) with 5 mM phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine. At the end of each reaction, cells were treated with 250 µl of 0.02 N HCl at 4°C for 20 min. The solution was collected, and CAMP concentrations were determined by radioimmunoassay, as previously described for rat proximal tubule epithelial cells in culture (42). Total protein was estimated by Bio-Rad DC protein assay, and CAMP accumulation was expressed as picomoles per milligram of protein.

Cytosolic calcium measurements. Intracellular free calcium in single T350K/AT₅ₐ cells was measured with the Ca²⁺ indicator fura 2-AM (pentaacetoxymethyl ester) using a video imaging system (S & M Microscope, Colorado Springs, CO), as described previously for vascular smooth muscle cells (31). Single cell images were acquired with an intensified charge-coupled device Dage 72 camera (Dage-MTI, Michigan City, IN) and processed though an IBM PC/AT computer using image-processing software (Image 1, Fluor; Universal Imaging, Westcheer, PA). Cells were grown on no. 1 glass coverslips in 6-well plates for 48 h. Cells were rinsed twice with Hanks' balanced salt solution (HBSS) and loaded with 50 µg fura 2-AM in 3 ml of HBSS buffer containing 20 mM HEPES, 1 mg/ml BSA, and 2.2 mg/ml glucose in a 37°C incubator in the presence of 5% CO₂ for 30 min. Cells were rinsed, and the emission signal from individual cells was measured. The excitation wavelengths were set at 340 and 380 nm. At the end of each experiment, the maximal emissions for calcium-bound fura 2 and free fura 2 were obtained by adding ionomycin (0.01 mM) and EGTA (0.15 mM), respectively. The intracellular calcium was calculated, as described previously (31). For these studies, data are expressed as the maximum increase in Ca²⁺ and occurred within 30 s of addition of ANG II.

Measurement of Na flux. Unidirectional AP-to-BL sodium flux was determined by a modification of methods previously described by our laboratory (41). Cells were grown to confluence on polycarbonate membrane inserts. Cells were rinsed with PBS, after which 500 µl of preincubation buffer containing (in mM) 136 NaCl, 5 NaHCO₃, 5 KCl, 4 Na₂HPO₄, 1.0 MgSO₄, 5.0 urea, 5.4 glucose, 2 glutamine, 296 mosmol/kg H₂O, pH 7.4, were added to AP, and 3 ml of the buffer were added to the BL side. The cells were incubated at 37°C, in the presence of 5% CO₂ for 1 h. Preincubation buffer was removed, and inserts were placed in the transport chamber. Aliquots of 100 µl were removed from the BL buffer from 1 to 20 min after initiation of transport. In preliminary studies, we found that 22Na transport was linear for up to 30 min after addition of agonist. 22Na was quantified by a liquid scintillation counter (Packard Tri-Carb), and 22Na transport from AP to BL buffer was determined.

Statistics. Results are expressed as means ± SE. Comparison between two groups was made by unpaired Student’s t-test. Statistical significance is defined as P < 0.05.

RESULTS

Stable expression of the AT₅ₐ receptor in OK cells. We utilized OK cells because most established cell lines derived from proximal tubules do not grow in a polar manner. OK cells are proximal tubule derived, as evidenced by preliminary studies (data not shown), which indicated 1) Na-dependent phosphate uptake, 2) parathyroid hormone- but not vasopressin-dependent CAMP responses, and 3) the presence of AP Na/H antiport, as indicated by pH-dependent Na uptake. To determine whether there was endogenous expression of ANG II receptors in OK cells, we performed 125I-ANG II binding studies on intact cells and RT-PCR analysis on total mRNA isolated from nontransfected OK cells (Fig. 1). 125I-ANG II binding in the presence of unlabeled ANG II demonstrated no displayable 125I-ANG II binding to intact cells (Fig. 1A), indicating undetectable levels of endogenous cell surface receptors in nontransfected OK cells. Furthermore, RT-PCR analysis using specific primers for ANG II AT₅ₐ receptor did not reveal PCR-amplifiable mRNA transcripts in wild-type OK cells (Fig. 1B), confirming our binding studies that AT₅ₐ receptors are absent in OK cells in culture. To understand the molecular and biochemical mechanisms of AT₅ₐ receptor function in proximal tubule cells, we expressed AT₅ₐ receptors in OK cells, and Geneticin-resistant stable cell lines were isolated. Screening for 125I-ANG II binding revealed numerous positive clones that displayed high level of receptor expression. We...
selected one representative clonal cell line for functional characterization and transcellular sodium transport studies, although the results presented here were confirmed in multiple cell lines expressing different levels of ANG II receptors. Prior to functional characterization, we sought to confirm that isolated cell lines expressed the recombinant AT1A receptor transcript. As shown in Fig. 1B, lane 5, RT-PCR analysis using specific primers for the AT1A receptor amplified a single PCR DNA product of 257 bp in size from the transfected OK cells. The size of this band corresponds to the expected size from inserted cDNA. Binding of 125I-ANG II at equilibrium (30 min, 22°C) to cells grown under nonpolarizing conditions is shown in Figs. 2 and 3. Competition binding studies of 125I-ANG II were performed at equilibrium using various concentrations (1 pM-10 µM) of nonradiolabeled ANG II. ANG II competed for the binding of 0.05 nM 125I-ANG II with a Kd of 5.27 nM, suggesting the expression of high-affinity receptors (Fig. 2). Scatchard analysis of the data demonstrated a Bmax of 6.02 pmol/mg protein. Competition binding experiments were performed to determine the ligand specificity of the transfected AT1A (Fig. 3). ANG II (Kd = 1.9 nM) and ANG III (Kd = 10.95 nM) effectively competed for binding of 0.05 nM 125I-ANG II to the receptor. Losartan, a competitive nonpeptide AT1 receptor antagonist, completely displaced 125I-ANG II binding with a Kd of 73.5 nM, confirming specific expression of transfected AT1A receptors. An AT2 receptor antagonist PD-123177 had no effect on 125I-ANG II binding to transfected receptors. High-affinity plasma membrane AT1A receptors have been shown to couple to
G proteins (21). The ability of plasma membrane AT1A receptors to couple to G proteins was determined by testing the ability of GMP-PNP, a nonhydrolyzable GTP analog, to lower the affinity of the receptor to ANG II. GMP-PNP inhibited the binding of 125I-ANG II to the receptor in a concentration-dependent manner (Fig. 4), indicating that AT1A couples to a G protein(s). To further characterize AT1A binding sites on AP and BL membranes of polarized cells, binding studies were performed on cells grown to confluency on permeable supports. Figure 5 demonstrates binding of 125I-ANG II to AP and BL surfaces of transfected OK cells. The studies were performed at 4°C to prevent over estimating Bmax due to receptor cycling. Both AP and BL 125I-ANG II binding were specific and saturable. One class of receptor was identified with comparable Kd on both sides: AP AT1A, Kd = 8.7 nM, Bmax = 3.33 pmol/mg protein; BL AT1A, Kd = 10.1 nM, Bmax = 5.50 pmol/mg protein.

AT1A receptor function in transfected OK cells. Coupling of ANG II to AT1 receptors results in the formation of d-myoinositol 1,4,5-trisphosphate and calcium release from intracellular stores (45). To determine whether transfected AT1A receptors were coupled to Ca2+, ANG II-mediated calcium transients were measured in T35OK/AT1A cells. Figure 6 shows that ANG II caused concentration-dependent intracellular calcium increases in AT1A receptor transfected OK cells. In a tissue-specific manner, ANG II receptors couple positively or negatively to cAMP (28, 35). To characterize functional coupling to cAMP in transfected cells, ANG II-mediated accumulation of cAMP in the presence of a phosphodiesterase inhibitor was measured. ANG II inhibited cAMP accumulation (Fig. 7); ANG II (100 nM for 7 min) decreased basal cAMP by 20%, from 13.2 to 10.7 pmol/mg protein, and decreased forskolin-stimulated cAMP by 62%, from 53.6 to 20.3 pmol/mg protein. Simultaneous incubation with the AT1 receptor-specific antagonist EXP-3174 (10 µM) reversed ANG II-mediated inhibition of cAMP. These results indicate that, in proximal tubule cells, AT1A receptors couple negatively to adenylate cyclase.

Previous studies have suggested that agonist-mediated receptor internalization is critical for AP receptor function in polarized proximal tubule epithelial cells (25, 40, 41). Figure 8 demonstrates that both AP and BL AT1A receptors underwent agonist-mediated internalization, as evidenced by time-dependent increases in acid-resistant labeled ligand. Internalization was rapid from each surface with 50% of bound ligand internalized in 7.9 min from AP surfaces and 2.1 min from BL surfaces.
To determine whether AP AT1A receptors were coupled to Na transport, transcellular 22Na transport was measured in OK cells exposed to ANG II. Figure 9 demonstrates that in T35OK/AT1A cells, activation of AP AT1A receptors resulted in time-dependent increases in transcellular 22Na transport. Compared with no additions, apical addition of ANG II (100 nM) to AT1A transfected cells increased 22Na transport by twofold at 20 min. ANG II induced comparable level of increases in 22Na transport at each time point tested. Apical addition of ANG II at concentrations of 1 and 100 nM increased 22Na transport. However, addition of concentrations of ANG II, 1 nM did not alter 22Na transport, and addition of ANG II, 100 mM did not inhibit 22Na transport. ANG II-induced increases in 22Na transport were blocked with EXP-3174, a nonpeptide receptor antagonist. To determine whether AT1A receptor-dependent 22Na transport was mediated by paracellular transport, the effect of ANG II (100 nM) on [3H]inulin leak was determined. In the absence of ANG II, AP-to-BL [3H]inulin leak (30 min) was 1.2 ± 0.2%, whereas BL-to-AP leak was 0.7 ± 0.1%. ANG II did not alter inulin leak: AP-to-BL [3H]inulin leak was 1.1 ± 0.2%, whereas BL-to-AP leak was 0.6 ± 0.1% (N = 6 in each group). To determine whether AT1A receptor-dependent 22Na transport was mediated through increases in Na/H exchange, transfected cells were treated with 100 µM of 5-(N,N-dimethyl)-amiloride (DMA). Figure 10 shows that addition of DMA prevented ANG II-dependent increases in 22Na transport, indicating that ANG II stimulation of 22Na transport is mediated through increases in Na/H exchange in AT1A transfected proximal tubule cells.

**DISCUSSION**

The major finding of our study is that, in contrast to the variable effects of ANG II on Na transport in vivo and in primary cultures of proximal tubules, ANG II resulted in time- and concentration-dependent increases in transcellular Na transport in OK cells transfected with rat AT1A receptors. These results are consistent with our earlier studies that indicated that ANG II, acting through AT1A receptors, increased transcellular Na transport in cultured rat proximal tubule epithelial cells (PTEC) (41). Although our results indicate that AT1A receptors increase transcellular Na transport, a number of studies have found inhibitory effects...
of ANG II on Na transport (12, 13, 24). The major transporter linked to ANG II in proximal tubules is the apical Na/H exchanger. Studies indicate a dose-dependent biphasic effect of ANG II on Na/H exchange: low concentrations stimulate and high concentrations inhibit transporter activity (27, 39). In addition to Na/H exchange, ANG II has been shown to regulate Na-K-ATPase in some studies (8, 19, 23). The key signaling enzymes linked to ANG II action in proximal tubule are PLC (42), PKC (15, 27, 34, 53), adenylate cyclase (33, 42), and PLA2 (15, 30). However, it is not clear which signaling enzyme(s) transduces ANG II action in proximal tubules. For example, ANG II-mediated increases in Na reabsorption have been attributed to the PLC/PKC signaling system linked to ANG II. In proximal tubules, PLA2 affects Na reabsorption has been associated with increases and decreases in Na/H exchange (1, 12, 20) and Na transport (4, 52), as well as Na-K-ATPase (6, 7). ANG II effects have also been attributed to decreases in cyclic AMP, but PKA inhibitors and exogenous cyclic AMP have not consistently been shown to alter ANG II-dependent regulation of Na/H exchange (11, 27). The role of PLA2 is also unclear, since PLA2 has been reported to activate or block ANG II regulation of Na transport and Na/H exchange (15, 30, 27).

There are a number of possible explanations for the reason we did not identify inhibitory effects of ANG II. 1) ANG II-dependent regulation of Na/H exchange has been utilized as a surrogate indicator of the effect of ANG II on transcellular Na transport. This assumption may not be correct, since proximal tubule Na transport can be regulated by apical Na entry, as well as basolateral Na extrusion by Na-K-ATPase. Signaling enzymes that regulate apical Na uptake can have opposite (or little) effect on basolateral Na extrusion. This is especially relevant for the PLC/PKC system. In short-term studies, PKC activators increase Na/H exchange (12) and inhibit Na-K-ATPase (6). The net effect on transcellular Na transport is, therefore, unpredictable. 2) The receptor mediating ANG II action is not always apparent from earlier studies. Recent data indicate that proximal tubule cells may express a number of ANG II receptor subtypes, including AT1A, AT1B, AT2, and AT4 receptors (16–18, 47, 55). Although ANG II binding to membrane preparations of proximal tubules has been reported (9, 10, 17), there are no reports of ANG II binding to proximal tubule cells. Lack of binding has been attributed to high levels of proteases, which degrade ligand and obscure binding. Alternatively, there may be a few receptors expressed in primary cultures, since specific binding has not been reported at low incubation temperatures, which would inactivate proteases. 3) In the absence of binding data, AT1A receptor dependency has been inferred from results using AT1 receptor blockers such as losartan. Although these results provide strong support for AT1 receptor dependency of ANG II action, the results may not be definitive because the agents bind to AT1A and AT1B receptors and, at high concentrations, AT2 receptors.

To determine specific effects of AT1A receptors on transcellular Na transport, we utilized an alternative strategy. We transfected OK cells, a cell line derived from proximal tubule epithelium, with AT1A receptor cDNA. The OK cell line we transfected did not express AT1A receptors (Fig. 1), and ANG II did not increase Na transport in nontransfected cells (data not shown). Although ANG II-dependent signaling and Na/H exchange have been reported in some OK cell lines (11, 29), to our knowledge, there are no reports of AT1A receptor message or of ANG II binding in OK cells. We found that AT1A receptors were expressed on both AP and BL membranes of transfected but not wild-type cells. Transfected AT1A receptors retained functional characteristics of native proximal tubule ANG II receptors. Binding was specific, saturable, and G protein dependent; both AP and BL receptors underwent internalization at approximately comparable rates. Transfected receptors manifest additional functions, including stimulation of Ca2+ signaling, negative coupling to adenylate cyclase, and amiloride-inhibitable transcellular Na transport.

ANG II-dependent calcium signaling in epithelial tissue was unanticipated, since concentrations of ANG II <1 µM have not been reported to increase cytosolic calcium in cultured PTEC (54). The lack of ANG II-dependent calcium signaling in epithelial tissue is in contrast to AT1A receptor signaling in nonpolar vascular smooth muscle cells. In nonpolar cells, AT1A receptors have been consistently linked to cytosolic calcium mobilization (45). Although our findings could be due to high expression of AT1A receptors in transfected OK cells, a more likely possibility is that too few AT1A receptors are expressed in PTEC to detect ANG II-dependent calcium signals using present methods.

Our data also substantiate the functional importance of AP AT1A receptors. Until recently, it had been assumed that BL ANG II receptors were functional, whereas AP receptors were not of physiological impor-
tance because 1) it had been assumed that urinary concentrations of ANG II were low, and 2) most of the well-studied signaling enzymes transducing ANG II actions were located BL. However, a number of observations have proven that these assumptions were incorrect. 1) Urinary ANG II levels are 10-fold greater than circulating ANG II levels (38, 44); 2) some AT1A receptor functions, including PLA₂ activation, are only activated by AP AT1A receptors (5); and 3) in PTEC, ANG II receptors are coupled to transcellular Na transport (41). The current study extends these observations and indicates that AP AT1A receptors acting through AP Na/H exchange mediate urinary ANG II-dependent increases in transtubular Na reabsorption.

In summary, the results of our studies indicate that the OK cells transfected with ANG II AT1A receptors stably express high-affinity receptors on both AP and BL surfaces, have similar functional characteristics of proximal tubule epithelial receptors, and positively coupled to Na transport. Increases in ANG II associated with activation of the renin-angiotensin system in response to volume depletion or in pathological conditions such as heart failure and cirrhosis could be mediated by the direct effects of ANG II acting on AT1A receptors in proximal tubules, as well as by the indirect effects of ANG II acting through changes in hemodynamics or aldosterone. These cells should provide a useful in vitro model to elucidate the molecular and biochemical mechanisms of AT1A expression and function in PTEC.

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