Role of internalization in AT1A receptor function in proximal tubule epithelium

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ANGIOTENSIN II (ANG II) has multiple effects on the cardiovascular system that are mediated through regulation of renal sodium reabsorption. Although ANG II-dependent changes in sodium reabsorption are mediated by ANG II-dependent increases in systemic and renal vascular tone and aldosterone secretion, ANG II directly regulates proximal tubule sodium reabsorption (17, 24). The proximal tubule effects of ANG II are transduced by angiotensin type 1 (AT1) receptors. In rats, two isoforms of AT1 receptors have been identified: AT1A and AT1B (13). All the effects of ANG II in the proximal tubule have been shown to be mediated by AT1A or AT1B receptors (9). For example, volume regulation is normal in AT1A knockout mice (6) but severely impaired in angiotensinogen knockout mice (19). These receptors are expressed on the apical and basolateral membranes of the proximal tubule (4, 5, 10). ANG II binding to apical or basolateral AT1A receptors results in inhibition of adenylate cyclase and increases in transcellular sodium reabsorption (16, 27).

Apical and basolateral membranes of the proximal tubule are composed of different lipids, signaling enzymes, and transporters. For example, basolateral membranes contain adenylate cyclase and sodium-potassium-ATPase, whereas apical membranes contain phospholipase (PL) A2 (3) and the NHE3 isoform of the sodium/hydrogen exchanger. Apical and basolateral AT1A receptors undergo internalization (2, 7). Earlier studies in cultured proximal tubules indicate that pharmacological agents that block cellular pathways of internalization decreased apical receptor-dependent increases in PLC, PLA2, and sodium transport (3, 22). In contrast, internalization blockers had no effect on basolateral AT1A receptor function (22). Because the pharmacological inhibitors of internalization interfere with cellular structures that regulate internalization, it was not known whether 1) AT1A receptors, rather than cell structures, regulate internalization-dependent signaling pathways and 2) internalization was required for apical and basolateral AT1A receptor function.

The AT1A receptor has been cloned, and the domains, which regulate receptor internalization, have been identified by mutation analysis. When internalization-mutant receptors were expressed in a number of different cells under nonpolarizing conditions, no identifiable changes in AT1A receptor function were reported (30). For example, noninternalizing AT1A mutants activate PLC and desensitize in transfected Chinese hamster ovary cells grown under nonpolarizing conditions. Because internalization pathways are not used for AT1A receptor function under nonpolarizing condi-
tions and because earlier pharmacological studies indicated that internalization pathways were critical to apical AT1A receptor-mediated increases in sodium transport in cultured polarized proximal tubule epithelium, the goal of this study was to determine the role of internalization on AT1A receptor signaling and sodium transport in polarized tissue. Because rat proximal tubule cells express AT1A and AT1B receptors, to determine AT1A receptor function without interference by other subtypes, we utilized an immortalized proximal tubule cell line, opossum kidney (OK), which does not normally express angiotensin receptors. OK cells were transfected with wild-type and mutant AT1A receptors to answer our questions.

MATERIALS AND METHODS

Materials. OK cells were obtained from Dr. John Raymond (Medical University of South Carolina). Dulbecco’s modified Eagle’s medium-Ham’s F-12 nutrient mixture, trypsin, gene-
ticin, bovine calf serum, and antibiotics were obtained from Life Technologies (Gaithersburg, MD). Tissue culture dishes, flasks, plastic ware, and Transwell cell culture inserts were purchased from Costar (Cambridge, MA). Radioiodinated ANG II was purchased from New England Nuclear (Boston, MA). ANG II was obtained from Sigma Chemical (St. Louis, MO). Candesartan was a generous gift from Astra Pharmaceu-
tical (Wayne, PA). All other molecular biology-grade chemicals were obtained from US Biochemical (Cleveland, OH), Fisher Scientific (Pittsburgh, PA), or Sigma Chemical.

Stable expression of the rat AT1A receptor in OK epithelial cells. The cloning and isolation of a genomic clone for the rat AT1A receptor, construction of the full-length receptor (coding for 359 amino acids), and construction of COOH-terminal truncated (TL314 and TK333) and deleted (Del 315–329) receptors into the pRe/CMV eukaryotic expression vector have been previously described (28, 30). Stable expression of the full-length AT1A receptor in OK cells has also been previously described (27). To develop mutant receptor-expressing cell lines, 50% confluent OK cells were transfected with 1–2 μg of plasmid DNA using the Lipofectamine method as described by the manufacturer (Life Science). Neomycin (G418)-resistant individual clones were isolated, and cell surface receptor expression was determined by 125I-ANG II binding studies. Cells stably expressing the receptor were maintained under a selection pressure of 200 μg/ml G418. Multiple clones expressing full-length and mutant receptors were isolated, and passages 6–30 were used. The results for individual clones were also confirmed in multiple clones.

ANG II receptor binding and internalization studies. Receptor transfected and nontransfected OK cells were grown to confluence on 6.5-mm-diameter, 0.4-μm-pore permeable Transwell membrane supports for differential binding to apical or basolateral surfaces (21). Confluence was confirmed by the development of a transepithelial resistance and barrier to transport of 3HJulin (21). 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 binding buffer or unlabeled ANG II were added to apical or basolateral surfaces, and then 270 μl of binding buffer containing 0.04–0.06 nM 125I-ANG II were added. Equilibrium binding was performed for 180 min at 4°C for apical or basolateral surface binding. Incubations were terminated by rapid removal of incubation solutions and addition of ice-cold PBS. Cells were washed three times with PBS and 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 bind-
ing. Specific binding was defined as total binding minus nonspecific binding (in the presence of unlabeled 1 μM ANG II). Nonspecific binding was <5% of total binding. Values were normalized to the amount of protein determined using the Bio-Rad DC protein assay. Results were analyzed using the computer software GraphPad Prism, and binding con-
stants were determined as described elsewhere (26).

To determine the rate of apical and basolateral receptor internalization, cells were grown to confluence on membrane inserts and assayed as described previously (28). Briefly, cells were washed twice with PBS, covered with binding buffer, and incubated at 37°C for 15 min. 125I-ANG II, in 30 μl of binding buffer, was added to apical or basolateral buffer (300 μl total volume) in a final concentration of 0.05 nM, and incubation continued at 37°C for 2, 5, 10, and 20 min. At each time point, inserts were chilled on ice, and radioactivity associated with noninternalized receptors was removed by two 40-s washes with 5 mM ice-cold acetic acid in 150 mM NaCl, pH 2.5. Radioactivity in the acid-sensitive and -insen-
sitive fractions was measured as described above.

The percentage of internalized receptors was plotted against time, curves were analyzed, and the half time to reach maximal level of internalization was determined using GraphPad Prism.

Measurement of cAMP accumulation. Cells were grown in 24-well Transwell plates in Dulbecco’s modified Eagle’s me-
dium-Ham’s F-12 nutrient mixture supplemented with 10% bovine calf serum, as previously described (27). Cells were washed twice with assay buffer containing 50 mM Tris, pH 7.4, 100 mM NaCl, 6 mM Na2HPO4, 4 mM KH2PO4, 0.25% bovine serum albumin, and 0.5 mg/ml bacitracin, and apical or basolateral surfaces were exposed to ANG II for 7 min in the same buffer (250 μl) with 5 mM 3-isobutyl-1-methylxan-
thine, a phosphodiesterase inhibitor. 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 radioimmunooassay as previously described for rat proximal tubule epithelial cells in culture (23). Total protein was estimated by Bio-Rad DC protein assay, and cAMP accumulation was expressed as picomoles per milligram of protein.

Measurement of sodium flux. Unidirectional apical-to-ba-
solateral sodium flux was determined by a modification of methods previously described by our laboratory (22). Cells were grown to confluence on polycarbonate membrane in-
serts. After cells were rinsed with PBS, 500 μl of preincuba-
tion buffer containing 136 mM NaCl, 5 mM NaHCO3, 5 mM KCl, 4 mM Na2HPO4, 1.0 mM MgSO4, 5.0 mM urea, 5.4 mM glucose, and 2 mM glutamine (296 mosM, pH 7.4) were added to the apical side and 3 ml of the buffer were added to the basolateral side. The cells were incubated at 37°C in the presence of 5% CO2 for 1 h. Preincubation buffer was re-moved, and inserts were placed in the transport chamber. 22Na (1 μCi/ml) was added to the apical chamber in transport buffer, which contained 128 mM NaCl, 5 mM NaHCO3, 5 mM KCl, 4 mM Na2HPO4, 1 mM MgSO4, 5 mM urea, 5.4 mM glucose, 2 mM glutamine, and 20 mM HEPES (305 mosM, pH 7.4). Basolateral surfaces were exposed to the same buffer without 22Na. Initiation of transport was defined as the time
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...when apical buffer was added. Aliquots of 100 μl were removed from the basolateral buffer 1–20 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 apical to basolateral buffer was determined.

Statistics. Values are means ± SE. Comparison between two groups was made by unpaired Student’s t-test and for more than two groups by one-way analysis of variance, followed by Tukey’s test for comparison of group means. Statistical significance is defined as P < 0.05.

RESULTS

cDNA encoding wild-type and mutated AT1A receptors were subcloned into the pRc/CMV mammalian expression vector and transfected into OK cells. Colonies resistant to G418 were selected and propagated. In preliminary studies, we sought to determine the minimum sequences required for cell surface expression of AT1A receptors. Transfection with TL306 failed to yield cell surface AT1A receptors, as indicated by kinetic analysis of binding: <10 fmol/mg for apical and basolateral surfaces. In contrast, transfection with TL314 resulted in stable expression of AT1A receptors.

As shown in Table 1, wild-type and TL314-mutated receptors bound ANG II with high affinity on apical and basolateral cell surfaces. On apical membranes, the number of binding sites tended to be less (477 ± 62 to 675 ± 46) and the binding affinity lower (1.25 ± 0.2 to 1.84 ± 0.4 nM) in the mutated than in the wild-type receptor (798 ± 63 fmol/mg and 3.32 ± 0.3 nM), although these values did not reach statistical significance. On basolateral membranes, the number of binding sites was similar (655 ± 54 to 1,065 ± 87) and the binding affinities were similar (1.46 ± 0.2 to 4.95 ± 0.4 nM) in mutated and wild-type receptors (865 ± 55 fmol/mg and 2.86 ± 0.4 nM). The level of cell surface receptor expression and binding affinity are comparable to values obtained for membrane preparations of rat proximal tubules (4, 5).

To determine whether wild-type and mutated AT1A receptors were internalized, we measured the appearance of acid-resistant radioactivity after addition of 125I-ANG II at 37°C. Figure 1 demonstrates internalization of wild-type and TL314-mutated receptors from apical and basolateral surfaces. As we reported earlier, >70% of apical and basolateral wild-type receptors were internalized within 20 min (27): 70 ± 5 and 82 ± 9% on apical and basolateral surfaces, respectively. In contrast, after 20 min, <15% of TL314-mutated apical (10 ± 2%, P < 0.01) and basolateral (14 ± 3%, P < 0.01) receptors were acid resistant and internalized. These data suggest that the cytosolic tail of the AT1A receptor is required for AT1A receptor internalization from apical and basolateral sites.

To determine whether mutated AT1A receptors were functional, we measured AT1A receptor coupling to adenylate cyclase (Fig. 2A) and to sodium transport (Fig. 2B). Figure 2 indicates that ANG II inhibited adenylate cyclase when added to apical (39 ± 7% vs. no additions) or basolateral (42 ± 8% vs. no additions) surfaces of cells transfected with wild-type receptor. There were time-dependent decreases in adenylate cyclase that were prevented by prior addition of the AT1A receptor blocker candesartan (95 ± 3 and 93 ± 4% for

Fig. 1. Time dependence of ANG II-stimulated receptor internalization from apical (AP) and basolateral (BL) surfaces of opossum kidney (OK)/angiotensin type 1A (AT1A) cells. OK cells stably expressing AT1A receptors were grown to confluence. Confluency was confirmed by development of transepithelial resistance and barrier to transport of [3H]inulin. 125I-ANG II was added to apical or basolateral buffers and incubated for 2–30 min at 37°C. At indicated times, surface-bound and internalized 125I was determined by acid washing. Internalization was calculated by expressing acid-resistant (internalized) radioactivity as a percentage of acid-resistant plus acid-susceptible (total binding) radioactivity. Values are means of triplicate determinations from 3 separate experiments.

Fig. 2. ANG II-mediated inhibition of cAMP (A) and stimulation of transcellular sodium transport (B) in AT1A receptor-transfected OK cells. OK cells stably expressing AT1A receptor constructs were grown to confluence on inserts. Apical and basolateral receptors were exposed to 100 nM ANG II. cAMP or apical-to-basolateral 22Na transport was determined as described in MATERIALS AND METHODS. Values are means ± SE of triplicate determinations from 3 separate experiments.
apical and basolateral surfaces, respectively, \( n = 4 \) in each group) and were maximal after 10 min. In contrast, ANG II did not inhibit adenylate cyclase from apical (94 ± 6\%) or basolateral (106 ± 8\%) sites in cells transfected with TL314-mutated receptor. Furthermore, higher concentrations of ANG II and longer duration of ANG II exposure were not associated with reductions in cAMP. As reported earlier, ANG II caused increases in transcellular sodium transport when added to apical (197 ± 16\%) or basolateral (214 ± 18\%) surfaces of cells transfected with the AT1A receptor (27). Increases in sodium transport were blocked by prior addition of candesartan: 106 ± 8 and 111 ± 3\% for apical and basolateral surfaces, respectively \([ n = 4 \) in each group, \( P = \) not significant (NS) vs. no additions]. In contrast, addition of ANG II did not result in increases in sodium transport when added to apical (106 ± 14\%) or basolateral (109 ± 11\%) surfaces of cells transfected with TL314-mutated receptors. The results indicated that removing the cytosolic tail of the AT1A receptor resulted in decreases in G protein coupling and sodium transport.

Because truncation mutation of the entire cytosolic tail altered G protein coupling, as well as internalization, we utilized two additional mutated receptors that do not contain putative G protein coupling sites: TK333 and Del 315–329. As shown in Table 1, both of these mutated receptors expressed high-affinity binding sites for ANG II. Figure 3 depicts internalization data for mutated receptors. Compared with wild-type receptors (64 ± 4 and 72 ± 5\% for apical and basolateral surfaces, respectively), both mutants displayed marked decreases in the amount internalized: for TK333, 32\% for the apical surface \((P < 0.01)\) and 27\% for the basolateral surface \((P < 0.01)\) and for Del 315–329, 30\% for the apical surface \((P < 0.01)\) and 24\% for the basolateral surface \((P < 0.01)\) after 20 min. Decreases in internalization from apical, but not basolateral, sites resulted in functional defects of apical, but not basolateral, AT1A receptors. Figure 4 indicates that basolateral exposure to ANG II resulted in reduction in cAMP in wild-type AT1A receptors (45 ± 6\% of no ANG II) as well as mutant AT1A receptors: 41 ± 5 and 56 ± 5\% for TK333 and Del 315–329, respectively \((both P = \) NS vs. wild-type AT1A). Figure 4B indicates that basolateral ANG II increased sodium transport by 208 ± 16\% in wild-type AT1A receptors and 177 ± 11\% and 188 ± 13\% in TK333 and Del 315–329 mutants, respectively \((both P = \) NS vs. wild-type AT1A). In contrast \((Fig. 4A)\), apical ANG II-dependent decreases in cAMP did not occur with either mutant receptor: 42 ± 6\% for wild-type AT1A receptor and 110 ± 10 and 104 ± 12\% for TK333 and Del 315–329, respectively \((both P < 0.01)\) vs. wild-type AT1A. In association with cAMP responses, Fig. 4B indicates that apical ANG II-dependent increases in sodium flux did not occur with either mutant receptor: 221 ± 14\% for wild-type AT1A receptor and 97 ± 7 and 94 ± 13\% for TK333 and Del 315–329, respectively \((both P < 0.01)\) vs. wild-type AT1A.

**DISCUSSION**

The results of our study indicate that 1) the COOH-terminal tail of the AT1A receptor contains sites that mediate agonist-dependent internalization, inhibition of adenylate cyclase, and increases in transcellular...
sodium transport and 2) receptor internalization is required for apical, but not basolateral, AT1A receptor inhibition of adenylate cyclase and increases in transcellular sodium transport.

The cytosolic tail of a number of cell surface receptors often regulates internalization. Whereas some receptors use a single segment, e.g., a NPXY motif, in the tail to drive internalization (β2-adrenergic low-density-lipoprotein receptors) (1, 31), other receptors use two motifs, one more proximal and the other distal, in the tail for internalization. Earlier studies indicate that two distinct regions in the cytosolic tail of the AT1A receptor are important for internalization. These motifs are at Leu316 to Tyr319, hydrophobic and aromatic residues on a putative α-helix, and Ser335 to Leu337, which contains an ST domain (11, 12, 29). AT1A receptors are internalized in polarized epithelial as well as nonpolarized tissues. Apical and basolateral receptor internalization occur at different rates (2). Our data indicate that apical and basolateral AT1A receptor internalization are regulated by the same motifs. When the 315–319 and 335–337 sites were removed (TL314 truncation), neither apical nor basolateral receptors internalized. Moreover, when the ST motif alone was removed (TK333 truncation), internalization was reduced by 80%. The ST site alone was not sufficient to drive internalization, because Del 315–329 caused an 80% reduction in internalization. These data indicate that the 316–329 and 335–337 motifs are required for internalization in epithelial tissues. The data also indicate that the cellular apparatus that interacts with AT1A receptors is the same for apical and basolateral receptors and that specific motifs on the receptor drive internalization and interaction with signal transduction pathways.

We also found that, in contrast to wild-type receptors, the TL314 truncation failed to inhibit adenylate cyclase or increase sodium transport when ANG II was added to the apical or the basolateral surface. We considered the possibility that this finding resulted from the aforementioned decreases in internalization but rejected this interpretation, because in earlier studies we showed that noninternalizing basolateral receptors were able to increase PLC and sodium transport (21, 22). Alternatively, the TL314 truncation could have been rendered nonfunctional as a result of a decrease in AT1A receptor coupling to G proteins, rather than decreases in internalization. A number of investigators have reported that the proximal region of the AT1A receptor cytosolic tail is important for G protein interactions. For example, Shirai et al. (25) found that a synthetic peptide containing residues 306–320 bound GTP analogs; Kai et al. (14) reported that a 304–316 residue inhibited ANG II-dependent GTPase activation, and Franzoni et al. (8) showed that the stretch between 306 and 320 was required to maintain the three-dimensional structure for AT1A receptors to interact with G proteins.

The Go protein coupling the AT1A receptor to signaling pathways has been identified. For example, Goq couples the receptor to PLC activation. Sano et al. (20) demonstrated that deletion mutations T309, T312, T313, and T314, but not T317, failed to activate PLC. They concluded that Tyr312, Phe313, and Leu314 were essential for coupling and activation of Goq. Goi couples AT1A receptors to inhibition of adenylate cyclase. However, there is limited information on the domain of the receptor that mediates this interaction. Whereas Shirai et al. (25) found that residues 306–320 were able to activate GTPase activity of Gi1 proteins, there are no reports on adenylate cyclase activity. Because the TL314 truncation failed to decrease adenylate cyclase while the Del 315–329 decreased adenylate cyclase, our data suggest that the 315–320 domain may be important for Gi1, as well as Gq, coupling.

The results also provide insight into AT1A receptor targeting to apical and basolateral membranes. Whereas internalization and/or G protein coupling domains regulate targeting of some receptors, AT1A receptor cell surface expression does not depend on these mechanisms. In this regard, the TL314 deletion mutation targeted to apical and basolateral surfaces (Table 1), even though the mutant did not internalize or signal. In other studies (data not shown), we found no expression of the TL306 mutation on apical or basolateral surfaces. These data indicate that sites between L306 and L314 regulate targeting.

Our study further defines the role of adenylate cyclase in mediating ANG II-dependent increases in proximal tubule sodium reabsorption. In micropipetted proximal tubules, Liu and Cogan (18) showed that ANG II decreased cAMP formation and increased sodium transport and that exogenous cAMP prevented this effect. Earlier we demonstrated that ANG II decreased adenylate cyclase and increased sodium transport in proximal tubule epithelial cells in culture and that exogenous cAMP blocked this effect as well (23). However, because both studies used high concentrations of exogenous cAMP, alternative interpretations were possible. Our present studies using a loss-of-function strategy indicate that cAMP decreases transduce AT1A receptor-mediated sodium transport in the proximal tubule. Mutations that uncoupled basolateral receptors from adenylate cyclase (TL314) prevented ANG II-mediated increases in sodium transport, whereas mutations that did not uncouple basolateral AT1A receptors from adenylate cyclase (TK333 and Del 315–329) resulted in inhibition of adenylate cyclase and increase in sodium transport.

The results also indicate that apical AT1A receptors internalize before interacting with Gi1, whereas basolateral AT1A receptors can activate Gi1 without internalization. In this regard, noninternalizing basolateral AT1A receptors containing the Gi1 site (TK333 and Del 315–329) decreased adenylate cyclase and increased sodium transport, but noninternalizing apical AT1A receptors were unable to inhibit adenylate cyclase or increase sodium transport. Earlier studies suggested that apical AT1A receptors internalize before activating signaling pathways, including PLC and PLA2 (3, 21). However, all of these studies utilized pharmacological agents, which were potentially nonspecific and, fur-
themselves, disrupted cellular internalization pathways, rather than the receptor. Our studies indicate that internalization pathways are driven by motifs on the receptor. The cellular internalization pathways and the mechanism by which AT1A receptors interact with these pathways are not known. Many receptors interact with an adapter/clathrin pathway and require dynamic or β-arrestin to internalize (15, 32). Neither of these pathways may be important for AT1A receptor internalization (32). The role of alternative pathways, such as the ubiquitin or caveolar pathways, in AT1A receptor internalization is not known. AT1A receptor internalization does not appear to be required for AT1 receptor function in nonpolarized tissue (30). For example, noninternalizing AT1 receptors activate PLC, calcium, mitogen-activated protein kinase, and other pathways (28). In nonpolarized tissue, G proteins and signaling enzymes are in the same membrane as AT1A receptors. In contrast, in the proximal tubule, adenylate cyclase is located in the basolateral membrane and is not opposed to apical AT1A receptors. Our results indicate that apical AT1A receptors communicate with basolateral adenylate cyclase and that this occurs by an internalization pathway. Either the receptor is internalized before interacting with G or G is internalized after interacting with AT1A receptors. It is not known whether G1 or G1 is internalized after interacting with AT1A receptors. Because adenylate cyclase is present in basolateral membranes of proximal tubule epithelium, it seems most reasonable to suggest that apical AT1A receptors are internalized before they interact with G1.

In summary, the results indicate that, in contrast to nonpolar cells, internalization pathways are important for AT1A receptor signaling and function in polarized proximal tubule epithelial cells. Apical and basolateral AT1A receptor expression does not require receptor internalization. Apical AT1A receptors undergo internalization before interacting with G proteins and inhibiting cAMP accumulation and increasing transcellular sodium transport. Basolateral AT1A receptors interact with G proteins and inhibit cAMP accumulation and increase sodium transport without internalizing.

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