AT_1_ receptor-mediated accumulation of extracellular angiotensin II in proximal tubule cells: role of cytoskeleton microtubules and tyrosine phosphatases

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AT_1_ receptor-mediated accumulation of extracellular angiotensin II in proximal tubule cells: role of cytoskeleton microtubules and tyrosine phosphatases. Am J Physiol Renal Physiol 291:F375–F383, 2006. First published February 14, 2005; doi:10.1152/ajprenal.00405.2005.—Long-term angiotensin II (ANG II) administration is associated with increased ANG II accumulation in the kidney, but intrarenal compartment(s) involved in this response remains to be determined. We tested the hypothesis that 1) extracellular ANG II is taken up by proximal tubule cells (PTCs) through AT_1_ receptor-mediated endocytosis, 2) this process is regulated by cytoskeleton microtubule- and tyrosine phosphatase-dependent mechanisms, and 3) AT_1_ receptor-mediated endocytosis of ANG II has a functional relevance by modulating intracellular cAMP signaling. In cultured PTCs, [125I]Tyr-labeled ANG II and fluorescein ANG II has a functional relevance by modulating intracellular cAMP phosphatases.

Inhibiting intracellular cAMP signaling. In cultured PTCs, [125I]Tyr-labeled ANG II and fluorescein ANG II were internalized in a time-dependent manner and colocalized with the endosome marker Alexa Fluor 594-transferrin. Endocytosis of extracellular ANG II was inhibited by the AT_1_ receptor blocker losartan (16.5 ± 4.6%, P < 0.01 vs. ANG II, 78.3 ± 6.2%) and by the tyrosine phosphatase inhibitor phenylarsine oxide (PAO; 30.0 ± 3.5%, P < 0.05 vs. ANG II). Intracellular ANG II levels were increased by −58% (basal, 229.8 ± 11.4 vs. ANG II, 361.3 ± 11.8 pg ANG II/mg protein, P < 0.01), and the responses were blocked by losartan (P < 0.01), the cytoskeleton microtubule inhibitor colchicine (P < 0.05), and PAO (P < 0.01), whereas depletion of clathrin-coated pits with hypertonic sucrose had no effect (356.1 ± 25.5 pg ANG II/mg protein, not significant). ANG II accumulation was associated with significant inhibition of both basal (control, 15.5 ± 2.8 vs. ANG II, 9.1 ± 2.4 pmol/mg protein, P < 0.05) and forskolin-stimulated cAMP signaling (forskolin, 68.7 ± 8.6 vs. forskolin + ANG II, 42.8 ± 13.8 pmol/mg protein, P < 0.01). These effects were blocked by losartan and PAO. We conclude that extracellular ANG II is internalized in PTCs through AT_1_ receptor-mediated endocytosis and that internalized ANG II may play a functional role in proximal tubule cells by inhibiting intracellular cAMP signaling.

Retaining endocytosed ANG II in proximal tubules is therefore important for renin-angiotensin system (RAS; including endogenous ANG II-dependent hypertension (14, 18, 24). Although cell surface AT_1_ receptor-mediated effects of ANG II in proximal tubules have been studied extensively, there is increasing evidence that PTCs may take up extracellular ANG II (both circulating and locally produced) via AT_1_ receptor-mediated endocytosis, which may be important for regulation of proximal tubular transport (11, 29, 30, 34, 35, 42). Nanomolar concentrations of ANG II have been reported in the glomerular filtrate (31), proximal tubular fluid (5, 20, 23, 31), and cortical interstitial fluid (25, 32). High levels of ANG II present in interstitial and intratubular fluid compartments combined with expression of abundant AT_1_ receptors in both apical and basolateral membranes provide PTCs with an ideal environment to promote cellular uptake through receptor-mediated endocytosis. For instance, increased whole kidney accumulation of circulating ANG II via AT_1_ receptor-mediated endocytosis has been consistently demonstrated in the contralateral (nonclipped) kidney of 2-kidney, 1-clip rats, a high-endogenous ANG II model of hypertension (13), along with kidneys of Ren-2 transgenic (20, 40) and ANG II-infused rats (38, 42, 44). However, the whole kidney approach does not allow identification of specific compartment(s) that may be responsible for intrarenal accumulation of ANG II in vivo.

Our group (42) recently demonstrated that increased intrarenal uptake of ANG II occurred primarily in renal cortical endosomes of ANG II-infused rats and was prevented by the AT_1_ receptor blocker candesartan. However, pharmacological blockers cannot distinguish between AT_1_ receptor subtypes, because there is ~95% genomic homology between AT_1A_ and AT_1B_ receptors (7). Most of AT_1_ receptor-mediated agonist endocytosis involves AT_1A_ receptors, whereas the role of AT_1B_ receptors remains unclear (7). To understand the role of AT_1_ receptor-mediated endocytosis in renal epithelial cells, opossum kidney (OK) epithelial cells and human embryonic kidney 293 cells (HEK-293) were transfected with AT_1A_ receptors (16, 34), but these cells do not express major components of the renin-angiotensin system (RAS; including endogenous AT_1A_ receptors), and therefore their physiological relevance remains uncertain. Recent evidence suggests that AT_1_ receptor-mediated endocytosis of extracellular ANG II is important not just for trafficking ANG II to the lysosomes for degradation

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and recycling of the receptors back to the membranes but also for full expression of the biological actions of ANG II in various cells (16, 29, 30, 34). For example, endocytosis of the ANG II-AT1 receptor complex is accompanied by increased phospholipase C- or phospholipase A2-mediated sodium flux and decreased cAMP production in renal epithelial cells (4, 29, 30, 34). These studies suggest that AT1A receptor-mediated endocytosis plays an important role in regulating PTC transport.

In the present study, we hypothesized that 1) extracellular ANG II is taken up by PTCs through AT1 receptor-mediated endocytosis; 2) receptor-mediated ANG II endocytosis contributes to increased intracellular accumulation of ANG II in PTCs; 3) blockade of receptor-mediated endocytosis by inhibitors of cell membrane cytoskeleton microtubules or tyrosine phosphatases prevents accumulation of ANG II in PTCs; and 4) AT1 receptor-mediated ANG II endocytosis plays a physiological role by regulating intracellular cAMP signaling. Using cultured rabbit PTCs derived from the S1 segment of proximal convoluted tubules, which express major components of the RAS (including AT1 and AT2 receptors), we demonstrated that AT1 receptor-mediated endocytosis of extracellular ANG II contributes to intracellular accumulation of ANG II in PTCs in vitro and plays an important role in the regulation of proximal tubule transport by modulating intracellular cAMP signaling.

MATERIALS AND METHODS

Materials. Cultured PTCs were obtained from American Type Culture Collection (vEPT; ATCC). These cells were initially derived from the S1 segment of rabbit kidney proximal convoluted tubules and have been shown to express electrolyte transporters as well as major components of the RAS, including angiotensinogen, renin, angiotensin-converting enzyme (ACE), and ANG II receptors (26, 27). Dulbecco’s modified Eagle’s medium, nutrient mixture, Ham’s F-12 (DMEM/F-12), trypsin, heat-inactivated fetal bovine serum (FBS), and the antibiotics penicillin and streptomycin were purchased from ATCC. Human Val5-ANG II, the radioligand [125I]Tyr-ANG II, and ANG II enzyme immunoassay kits were obtained from Biochrom/Peninsula Laboratories. cAMP enzyme immunoassay kits were purchased from R&D Systems. The AT1 receptor antagonist losartan was a gift from Merck Pharmaceuticals, and the AT2 receptor antagonist PD-123319 was donated by Pfizer. AT1 receptor small-interference RNA (siRNA) and rabbit polyclonal AT1 receptor antibody targeting the NH2-terminal extracellular domain of the human AT1 receptor (N-10), scrambled siRNA, and transfection reagents were purchased from Santa Cruz Biotechnology. Western blot signals were detected using enhanced chemiluminescence (Amersham) and analyzed using a microcomputer imaging device with a digital camera (MCID, Imaging Research, Ontario, Canada).

AT1 receptor-mediated endocytosis of extracellular ANG II. To determine whether AT1 receptors are internalized by PTCs when exposed to extracellular ANG II, the cells were incubated with 100 pmol [125I]Tyr-ANG II for 2, 5, 10, 15, or 30 min at 37°C alone or in the presence of the AT1 receptor blocker losartan (10 μM) or the specific tyrosine phosphatase inhibitor PAO (1 μM), both known to inhibit AT1A receptor endocytosis (9, 12, 30). At each time point, incubations were stopped by washing the cells twice with ice-cold PBS to remove free radioligands from the medium. Acid-sensitive (noninternalized) and -insensitive radioactivity (internalized) were separated by washing the cells twice with 5 mM ice-cold acetic acid buffer in 150 mM NaCl, pH 2.5. Radioactivity was counted and the percentage of internalized or noninternalized receptors analyzed (2, 12, 34).

Effects of AT1 and AT2 receptor blockade on intracellular accumulation of ANG II. To determine the role(s) of AT1 receptor-mediated ANG II endocytosis, PTCs were treated with vehicle (serum-free medium), ANG II (Val5-ANG II; 1 nM), ANG II plus losartan (10 μM), or ANG II plus PD-123319 (10 μM) for 60 min at 37°C. After treatment, the medium was removed and the cells washed twice with ice-cold PBS and then twice with ice-cold acid buffer (5 mM acetic acid, 150 mM NaCl, pH 2.5) to remove any cell membrane-bound ANG II (2, 12, 16, 34). ANG II was extracted from PTCs in a buffer containing 20 mM Tris·HCl, 1 mM EDTA, 5 mM EGTA, 5 mM mercaptoethanol, 50 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A and measured using a sensitive and specific ANG II enzyme immunoassay kit (Biochem/Peninsula).

Effects of inhibitors of clathrin-coated pits, cytoskeleton microtubules, and tyrosine phosphatase on intracellular ANG II accumulation. To determine the role(s) of clathrin-coated pits, cytoskeleton microtubules, and tyrosine phosphatases in AT1 receptor-mediated ANG II endocytosis in PTCs, the cells were treated with serum-free medium alone, ANG II (1 nM), ANG II plus 400 mM sucrose, which depletes clathrin-coated pits (2, 9, 10), ANG II plus the tyrosine microtubule inhibitor colchicine (1 μM) (3, 8, 29), or ANG II plus the tyrosine phosphatase inhibitor PAO (1 μM) to block AT1 receptor-mediated endocytosis (9, 12, 30). PAO is an established tyrosine
RESULTS

Expression of AT1 receptors in rabbit proximal tubule cells. As shown in Fig. 1A, radioreceptor binding assays using [125I]Tyr-ANG II showed that PTCs express a single class of ANG II receptor binding. Scatchard analysis suggests that these cells bound [125I]Tyr-ANG II with an apparent Kd of 6.8 ± 2.1 nM, and the Bmax averaged 1,012 ± 28 fmol/mg protein. The AT1 receptor predominates in these cells because losartan (10 μM), a competitive AT1 receptor blocker, displaced over 90% of total ANG II receptor binding, whereas the AT2 receptor-selective blocker PD-123319 (10 μM) inhibited <10% of binding. Next, we used two approaches to confirm that PTCs express AT1 receptors. First, with the use of a rabbit anti-AT1 receptor polyclonal antibody against the NH2-terminal extracellular domain of the human AT1 receptor (11, 17, 42), Western blot detected a single protein band of ~42 kDa (Fig. 1B). The AT1 receptor protein we detected is consistent with the AT1 receptor reportedly expressed in the rat kidney (15). In previous studies, pretreatment of samples with an AT1 receptor-selective antigen blocking peptide (Santa Cruz) before running the Western blot confirmed the specificity of the AT1 receptor-selective antigen blocking peptide (Santa Cruz) before running the Western blot confirmed the specificity of the AT1 receptor-selective antigen blocking peptide (Santa Cruz). Second, a scrambled AT1 siRNA had no effect. **P < 0.01 vs. control.

AT1 receptor-mediated endocytosis of extracellular ANG II. AT1A receptors have been shown to internalize after exposure to extracellular ANG II in OK or HEK-293 cells, two renal epithelial cell lines commonly used for transfection of AT1A receptors because they do not express endogenous receptors (16, 34). Using both the radioligand [125I]Tyr-ANG II and fluorescence-conjugated ANG II, we determined whether extracellular ANG II is internalized after binding to cell surface AT1 receptors in rabbit PTCs and whether losartan or PAO blocked its endocytosis. Figure 2 shows that extracellular ANG II was internalized in a time-dependent manner, with close to 80% internalized within 30 min of incubation (78.3 ± 6.2%).
Time-dependent endocytosis of extracellular ANG II was almost completely inhibited by blocking of AT1 receptors with losartan (16.5 ± 4.6%, P < 0.01) or inhibiting tyrosine phosphatase with PAO (30.0 ± 3.5%, P < 0.05). Thirty minutes after incubation, fluorescence microscopy shows that fluorescein-labeled ANG II was localized to the cytoplasm of the cells (Figs. 3, A and C), where it colocalized with Alexa Fluor 594-labeled transferrin, an endosomal marker (Figs. 3, D and F). These results suggest that after endocytosis, ANG II is trafficked mainly into endosomes of PTCs.

Effects of AT1 and AT2 receptor blockade on accumulation of ANG II. Our group (42, 44) has previously shown that extracellular ANG II is accumulated in the rat kidney via AT1 receptor-mediated endocytosis after long-term ANG II infusion. High levels of ANG II were later demonstrated in isolated renal cortical endosomes and intermicrovillar clefts of ANG II-infused rats (42). In the present study, intracellular ANG II levels in PTCs were measured using an ANG II enzyme-linked immunoassay (Biochem/Peninsula Laboratories). The assay demonstrated high levels of sensitivity and can detect up to 20 pg ANG II/ml or 2 pg ANG II per well of six-well-plates (intra-assay variation <5%; interassay variation <14%). There is 100% cross-reactivity with ANG II and Val5-ANG II, but only 0.5% with ANG I. Basal ANG II levels in PTCs averaged 229.8 ± 11.4 pg ANG II/mg protein (Fig. 4). Incubation of PTCs with Val5-ANG II (1 nM) for 1 h at 37°C increased intracellular ANG II by ~58% (361.3 ± 11.8 pg ANG II/mg protein, P < 0.001 vs. basal). Blockade of AT1 receptors with losartan (10 μM) effectively prevented increased ANG II (254.3 ± 8.8 pg ANG II/mg protein, P < 0.001 vs. ANG II). Losartan alone did not alter basal ANG II levels (220.5 ± 6.9 pg ANG II/mg protein, not significant vs. basal). Interestingly,
cordadministration of PD-123319 with ANG II also slightly reduced intracellular ANG II to the level seen with ANG II alone (287.3 ± 26 pg ANG II/mg protein, P < 0.05 vs. ANG II). These data suggest that both AT1 and AT2 receptors mediate endocytosis of extracellular ANG II in PTCs, but it is AT1 that predominated.

Effects of inhibitors of clathrin-coated pits, cytoskeleton microtubules, and tyrosine phosphatases on ANG II accumulation. Clathrin-coated pits, cytoskeleton microtubules, and tyrosine phosphatases have been separately shown to play an important role in AT1A receptor-mediated endocytosis in vascular smooth muscle cells (VSMCs) (2), Chinese hamster ovary (CHO) cells transfected with AT1A receptors (9), or rat PTCs (29, 30). However, it is not clear whether AT1-mediated endocytosis contributes to accumulation of extracellular ANG II in rabbit PTCs via a similar mechanism. Figure 5A shows that incubation of PTCs at 4°C with Val(5)-ANG II, which inhibits receptor-mediated endocytosis, prevented the AT1-mediated increase in intracellular ANG II accumulation (ANG II, 367.6 ± 18.9 pg ANG II/mg protein; ANG II plus 4°C, 213.2 ± 17.7 pg ANG II/mg protein, P < 0.001). Unexpectedly, depletion of clathrin-coated pits with 400 mM sucrose did not prevent intracellular ANG II accumulation (356.1 ± 25.5 pg ANG II/mg protein, not significant vs. ANG II). As shown in Fig. 5B, both colchicine, an inhibitor of cytoskeleton microtubule-mediated protein trafficking (8, 29), and PAO, an inhibitor of tyrosine phosphatases and receptor-mediated endocytosis (12, 29), prevented intracellular ANG II accumulation (ANG II + colchicine, 266.9 ± 28 pg ANG II/mg protein, P < 0.05 vs. ANG II; ANG II + PAO, 190.8 ± 17.9 pg ANG II/mg protein, P < 0.001 vs. ANG II). These results suggest that cytoskeleton microtubules and tyrosine phosphatases, but not clathrin-coated pits, mediate extracellular ANG II accumulation in PTCs.

Effects of AT1 receptor-mediated endocytosis of extracellular ANG II on intracellular cAMP production. Previous studies suggested that AT1 (or AT1A) receptor-mediated endocytosis of extracellular ANG II may play an important role in full expression of biological actions of ANG II in various cells (29, 30, 34). To determine whether AT1-mediated endocytosis of extracellular ANG II has a functional relevance, we measured cAMP production in PTCs using an enzyme-linked immunoassay (R&D). AT1-mediated ANG II endocytosis was blocked by the AT1 receptor blocker losartan (10 μM) or the tyrosine phosphatase inhibitor PAO (1 μM). Figure 6A shows time-dependent inhibition of cAMP production by ANG II, which peaked at 30 min. At 30 min, ANG II inhibited basal cAMP production (basal, 15.5 ± 2.8 pmol/mg protein; ANG II, 9.1 ± 2.4 pmol/mg protein, P < 0.05). The effect of ANG II on basal cAMP production was reversed by losartan (33.3 ± 6.3 pmol/mg protein, P < 0.05) and PAO (22.5 ± 3.6 pmol/mg protein, P < 0.05) to levels significantly above control (Fig. 6B). As shown in Fig. 7, the adenylyl cyclase activator forskolin (10 μM) markedly increased intracellular cAMP levels (68.7 ± 8.6 pmol·mg⁻¹·protein⁻¹, P < 0.01) (Fig. 7). ANG II significantly reduced forskolin-induced increases in cAMP (42.8 ± 13.8 pmol/mg protein, P < 0.05 vs. forskolin alone). Both losartan and PAO, which inhibit AT1-mediated endocytosis, blocked ANG II-induced inhibition of forskolin-mediated increases in intracellular cAMP production (ANG II + forskolin + losartan, 54.3 ± 14.7 pmol/mg protein, P < 0.05 vs. ANG II + forskolin; ANG II + forskolin + PAO, 36.3 ± 5.9 pmol/mg protein, P < 0.05 vs. ANG II + forskolin) (Fig. 7B). Losartan alone also slightly increased cAMP production by ~15% above control (P < 0.05), whereas PAO alone had no effect. PD-123319 had no effect on ANG II-induced inhibition of the forskolin-mediated increase in cAMP production (22.5 ± 5.6 pmol/mg protein, not significant vs. ANG II +
ANG II endocytosis, our results suggest that increased endo-
colchicine, or the tyrosine phosphatase inhibitor PAO; and losartan, cold (4°C), the cytoskeleton microtubule inhibitor
of AT1 receptor-mediated endocytosis with various endocy-
totic inhibitors prevented AT1-mediated intracellular
inhibitor of cytoskeleton microtubules, and PAO, an inhibitor
receptor-activated signaling. Because colchicine, a selective
intracellular ANG II exerts a functional effect on ANG II
of circulating ANG II ex vivo and that after endocytosis,
ANG II in PTCs plays an important role in renal accumulation
of extracellular ANG II may affect intracellular cAMP signaling in PTCs.

**DISCUSSION**

The present study produced three key findings, namely, 1) cultured PTCs derived directly from the S1 segment of rabbit proximal tubules expressed predominantly AT1 receptor protein equivalent to AT1A receptors in rodents; 2) incubating these cells with Val5-ANG II significantly increased intracellular ANG II accumulation, which was blocked by inhibition of receptor-mediated endocytosis with the AT1 receptor blocker losartan, cold (4°C), the cytoskeleton microtubule inhibitor colchicine, or the tyrosine phosphatase inhibitor PAO; and 3) AT1 receptor-mediated endocytosis of extracellular ANG II has a functional role, as indicated by the finding that blockade of AT1 receptor-mediated endocytosis with various endocytotic inhibitors prevented ANG II-induced inhibition of basal and forskolin-stimulated intracellular cAMP production. These results suggest that AT1-mediated endocytosis of extracellular ANG II in PTCs plays an important role in renal accumulation of circulating ANG II ex vivo and that after endocytosis, intracellular ANG II exerts a functional effect on AT1 receptor-activated signaling. Because colchicine, a selective inhibitor of cytoskeleton microtubules, and PAO, an inhibitor of tyrosine phosphatases, prevented AT1-mediated intracellular ANG II accumulation, our results suggest that increased endo-
cytosis of extracellular ANG II in PTCs is cytoskeleton micro-
tubule- and tyrosine phosphate-dependent. By contrast, hyper-
osmotic sucrose (400 mM), which inhibits GPCR endocytosis in VSMCs or CHO cells by depleting clathrin-coated pits (9, 28), failed to prevent intracellular ANG II accumulation, indicating that clathrin-coated pits do not play a significant role in AT1 receptor-mediated ANG II accumulation in rabbit PTCs.

The present study provides evidence that AT1-mediated ANG II endocytosis plays an important role in high intracellu-
lar accumulation of ANG II in PTCs in vitro and, by implication, that this process may also occur in PTCs ex vivo. Investigators in our group and others have previously shown that circulating ANG II accumulates in the kidney of ANG II-infused rats via an AT1-mediated mechanism(s) (36, 38, 42, 44); however, the cellular location of ANG II accumulation in the kidney as well as the mechanisms involved have not been determined. ANG II levels in the kidney are reportedly several thousand times higher than the circulating peptide, which leads to the hypothesis of compartmentalization of ANG II synthesis and/or release within the kidney (5, 23). Indeed, early studies reported nanomolar concentrations of ANG II in intrarenal fluid compartments, including the glomerular filtrate (31), proximal tubular fluid (5, 31), and renal cortical interstitial fluid (25, 32) compared with femto- to picomolar levels in the circulation. However, when van Kats et al. (35) infused 125I-labeled ANG II into pigs and measured labeled peptide levels in different cellular fractions of the kidney homogenates, they found that most 125I-ANG II is cell-associated due to AT1 receptor-mediated endocytosis. We previously measured internalized AT1A receptors and ANG II in isolated renal cortical endosomes and intermicrovillar clefts of ANG II-infused rats and found that AT1A receptor antibody binding more than doubled, whereas ANG II levels were 5–10 times higher in endosomes and more than doubled in intermicrovillar clefts compared with control (42). Because coadministration of the AT1 receptor blocker candesartan prevented accumulation of extracellular ANG II in endosomes and intermicrovillar clefts, we interpreted these findings as an AT1A-mediated response. However, it should be emphasized that all previous studies

![Fig. 6. Effects of blockade of AT1-mediated ANG II endocytosis by losartan and PAO on basal intracellular cAMP responses to ANG II in PTCs. A: time-dependent inhibition of cAMP production by ANG II (1 nM), which peaked at 30 min. B: at 30 min, ANG II (1 nM) attenuated basal cAMP production and that losartan and PAO reversed ANG II-induced inhibition of basal cAMP production. *P < 0.05 vs. control. #P < 0.05 vs. ANG II.](http://ajprenal.physiology.org/)

![Fig. 7. Effects of blockade of AT1-mediated ANG II endocytosis by losartan and PAO on forskolin-stimulated cAMP production in PTCs 30 min after exposure to the agonist and/or blockers. Note that forskolin-stimulated cAMP production and ANG II (1 nM) significantly attenuated forskolin-stimulated cAMP production. Both losartan and PAO reversed ANG II-induced inhibition of forskolin-increased cAMP production, whereas PD-123319 did not. FKL, forskolin. *P < 0.05 vs. control. +P < 0.05 vs. ANG II. #P < 0.05 vs. FKL. &P < 0.05 vs. ANG II + FKL.](http://ajprenal.physiology.org/)
were performed in whole kidney tissue, and therefore it is not possible to determine the cellular sites responsible for increased accumulation of extracellular ANG II in the kidney after long-term ANG II infusion.

In the present study, we used cultured rabbit PTCs as a tool to determine the contribution of AT1-mediated endocytosis of extracellular ANG II to intracellular accumulation of ANG II and study the potential role(s) of clathrin-coated pits, cytoskeleton microtubules, and tyrosine phosphatases in AT1-mediated ANG II accumulation in PTCs in vitro. Use of these cells as a model offers several advantages over the whole kidney approach in vivo. These cells express all necessary components of the RAS, including ANG II receptors, and respond to ANG II stimulation by activating intracellular signaling, commonly associated with PTC function (26, 27, 43). Although proximal tubules can be isolated for measurement of ANG II, the procedures are laborious and time-consuming, and the reagents used for isolation and purification would likely alter ANG II formation and degradation. The main disadvantage with using whole kidney homogenates is perhaps that it is not possible to study the cellular mechanisms involved beyond the role of AT1 receptors. In the present study, we first demonstrated that AT1 (equivalent to human AT1 and rodent AT1A) receptor proteins are expressed in these PTCs by Western blot, using rabbit anti-AT1 receptor antibodies raised against the NH2-terminal or cytosolic domains of the receptor (11, 17, 42) and an AT1 receptor-selective siRNA. We then confirmed that incubating these cells with 125I-ANG II induced ~80% internalization of the peptide within 30 min of exposure and that this was blocked by losartan, pointing to an AT1 receptor-mediated mechanism. This phenomenon has been demonstrated previously in OK cells transfected with AT1A receptors, although basal and internalized ANG II were not measured (33). We therefore extended previous studies by measuring intracellular ANG II before and after exposure to extracellular ANG II to promote receptor-mediated endocytosis. On the basis of cell number and protein content per well and ANG II concentrations, we can estimate basal endogenous ANG II content in these cells as well as the relative contribution of AT1-mediated endocytosis of extracellular ANG II to intracellular ANG II accumulation in PTCs. There are ~10^6 cells or 360 μg protein in each well of a six-well plate when they have grown to 80% confluence. The basal concentration of ANG II is close to 200–250 pg/mg protein, which gives a calculated basal ANG II content of ~70–90 pg/10^6 cells. Intracellular ANG II content would increase by 50–70% to ~110–150 pg/10^6 cells, primarily due to AT1-mediated endocytosis.

Whether AT2 receptors mediate ANG II endocytosis and its subsequent intracellular signaling is not known (7, 9, 16, 45). Hein et al. (16) showed that unlike AT1 receptors, AT2 receptors transiently or stably expressed in HEK-293 cells do not internalize when they are stimulated by ANG II. As shown in Figs. 1 and 4, however, we found that rabbit PTCs also express low levels of endogenous AT2 receptors and that the AT2 receptor antagonist PD-123319 partially inhibited intracellular accumulation of extracellular ANG II, suggesting that endogenous AT2 receptors perhaps play a minor role in mediating ANG II endocytosis in rabbit PTCs. Indeed, AT2 receptors have been reported to mediate different biological effects in various renal cells (7).

The cellular mechanisms that regulate AT1 (or AT1A) receptor-mediated endocytosis are complex and often cell type specific. There are two recognized pathways for GPCR endocytosis, the classic clathrin-dependent and non-clathrin-dependent pathways (1, 9, 28). The most commonly cited pathways for GPCR-mediated endocytosis include clathrin-coated pits, β-arrestin and/or dynamin proteins, β-adaptin, and G protein-coupled receptor kinases (1, 9, 28). Because discussion of the role(s) of individual pathways in AT1A receptor-mediated endocytosis is beyond the scope of the present study, we focused on the potential role(s) of three important pathways in mediating intracellular ANG II accumulation in PTCs. Clathrin-coated pits or vesicles, interacting with β-arrestin and/or dynamin proteins, are widely credited with endocytosis of epidermal growth factor (9, 35) and β2-adrenergic receptors (9) and also with AT1A receptors in CHO or HEK-293 cells stably transfected with the mutant receptors (16, 33). We questioned whether clathrin-coated pits play a role in AT1-mediated ANG II accumulation in PTCs. Our group previously showed that in the ANG II-infused rat kidney, ANG II accumulated in renal cortical endosomes, where it colocalized with AT1A receptors (42); yet we could not determine whether clathrin-coated pits play any role in intracellular trafficking of ANG II/AT1 receptor complex to the endosomes in vivo. In the present study, we found that pretreating PTCs with hyperosmotic sucrose (400 mM), which is commonly used to deplete clathrin-coated pits (2, 9, 35), did not significantly prevent receptor-mediated intracellular accumulation of ANG II, suggesting that non-clathrin endocytic pathways may play an important role in PTCs. Non-clathrin endocytic pathways also can deliver molecules to classic endocytic compartments, such as endosomes, and to other intracellular compartments, such as the Golgi apparatus and endoplasmic reticulum (28). Schelling et al. (29) demonstrated that in cultured rat PTCs, blocking receptor-mediated endocytosis with the cytoskeleton microtubule inhibitor colchicine or PAO, a tyrosine phosphatase-selective inhibitor, completely eliminated apical ANG II-induced phospholipase C (PLC)-mediated intracellular inositol 1,4,5-trisphosphate (IP3) signaling and 22Na transport. In the present study, we demonstrated that colchicine and PAO completely prevented AT1-mediated intracellular ANG II accumulation in PTCs, supporting the hypothesis that AT1-mediated endocytosis of extracellular ANG II in PTCs is cytoskeleton microtubule-dependent and requires activation of tyrosine phosphatases.

How cytoskeleton microtubules or tyrosine phosphatases could modulate AT1-induced intracellular accumulation of extracellular ANG II in PTCs remains to be determined. Cytoskeleton microtubules are polarized cytoplasmic structures extending from the perinuclear region toward the periphery of the cell (3, 6, 19). Cytoskeleton microtubules, acting through the dynein activator protein dynactin, play an important role in cytoplasmic trafficking of viruses, solutes, or proteins from early endosomes to late endosomes or lysosomes and from the endoplasmic reticulum to the Golgi apparatus inside mammalian cells (3, 6, 19). PTCs are polarized epithelial cells with their apical membrane facing the tubular lumen and their basolateral membrane touching the peritubular capillaries. Solutes, amino acids, peptides, and glucose are transported into cells via receptor-mediated endocytosis or by various transporters (3, 6, 21). It is likely that colchicine prevented intra-
cellular ANG II accumulation by inhibiting cytoplasmic trafficking of the peptide after endocytosis. In VSMCs, disruption of cytoskeleton microtubules with nocodazole blocked AT<sub>1</sub> receptor trafficking into caveolae/lipid rafts (45). By contrast, PAO may block AT<sub>1A</sub>-mediated ANG II endocytosis via a different mechanism. PAO is a general inhibitor of tyrosine phosphatases that has been widely used to study AT<sub>1A</sub> receptor endocytosis (12, 30), but it is not clear which specific tyrosine phosphatase it inhibits and how it inhibits AT<sub>1A</sub> receptor endocytosis. Previous studies have shown that PAO inhibits not only AT<sub>1A</sub> receptor endocytosis (12, 30) but also other GPCR endocytosis (33, 36). Thus PAO may not act specifically at the receptor level and, instead, inhibits receptor endocytosis by targeting the endocytic machinery such as arrestins, dynamins, or cytoskeleton microtubules. It is also likely that PAO may inhibit one of tyrosine phosphatases that play a role in GPCR endocytosis. Nevertheless, because PAO inhibits protein tyrosine phosphatases and therefore induces protein tyrosine dephosphorylation, our results suggest that tyrosine phosphatases and/or tyrosine dephosphorylation are involved in AT<sub>1A</sub>-receptor-mediated intracellular accumulation of extracellular ANG II in proximal tubule cells. Further studies are required to identify which specific protein tyrosine phosphatase regulates AT<sub>1A</sub> receptor endocytosis and elucidate the cellular mechanisms involved.

Our results show that AT<sub>1</sub>-mediated endocytosis of extracellular ANG II may play a functional role in regulating proximal tubular sodium transport. In the present study, increased intracellular accumulation of extracellular ANG II via AT<sub>1</sub> receptor-mediated endocytosis was associated with decreased basal and forskolin-stimulated intracellular cAMP production. Losartan and PAO inhibited AT<sub>1</sub>-mediated ANG II endocytosis in PTCs, and both prevented the effects of ANG II on intracellular cAMP production, indicating that internalized ANG II does indeed play a functional role in PTC function. Alternatively, because coadministration of losartan or PAO with ANG II increased cAMP production to the levels that were significantly higher than control or ANG II alone (Fig. 6B), other mechanisms unrelated to AT<sub>1</sub>-mediated ANG II endocytosis may be involved. There is evidence that receptor-mediated ANG II endocytosis is important not just for transporting the ligand to the lysosomes for destruction and recycling the receptors back to the cell surface and that receptor-mediated ANG II endocytosis may be important in regulating biological actions of ANG II in PTCs. Schelling et al. (29, 30) demonstrated that endocytosis of the ANG II-AT<sub>1</sub> receptor complex activated PLC-IP<sub>3</sub> signaling, increased sodium flux, and decreased cAMP signaling in cultured rat PTCs. Becker et al. (4) showed that AT<sub>1</sub> receptor-mediated endocytosis was associated with increased phospholipase A<sub>2</sub> activity and sodium flux in LLC-PK cells expressing rabbit AT<sub>1</sub> receptors. Thkekumkara and Linas (34) reported that in OK cells, apical membrane AT<sub>1</sub>A receptors were internalized before they interact with G proteins, leading to inhibition of cAMP signaling. Accordingly, our finding that inhibition of AT<sub>1</sub> receptor-mediated endocytosis of extracellular ANG II blocked intracellular ANG II accumulation, and therefore ANG II-induced inhibition of cAMP signaling in PTCs, is consistent with these previous observations.

In summary, we have demonstrated in cultured rabbit PTCs, which express endogenous AT<sub>1</sub> receptors, that 1) intracellular ANG II levels increase significantly when cells are exposed to extracellular ANG II; 2) increased intracellular ANG II accumulation is inhibited by the AT<sub>1</sub> receptor antagonist losartan, the cytoskeleton microtubule inhibitor colchicine, or the tyrosine phosphatase inhibitor PAO; 3) depletion of clathrin-coated pits with hyperosmotic sucrose has no effect on intracellular ANG II accumulation; and 4) inhibition of AT<sub>1</sub> receptor-mediated intracellular ANG II accumulation blocks ANG II-inhibited cAMP production. These results suggest that AT<sub>1</sub> receptor-mediated endocytosis of extracellular ANG II in PTCs contributes to increased intrarenal ANG II accumulation in vivo and also plays a functional role in the regulation of proximal tubule cell function by regulating intracellular cAMP signaling.

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REFERENCES


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