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AT_1 receptor-mediated accumulation of extracellular angiotensin II in proximal tubule cells: role of cytoskeleton microtubules and tyrosine phosphatases

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Abstract

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, [^{125}I]Tyr-labeled ANG II and fluorescein labeled-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 hyperosmotic 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.

Keywords

kidney; receptor-mediated endocytosis

Proximal tubule cells (PTCs) are the major target of a dual vasoactive peptide angiotensin II (ANG II), acting as both a circulating endocrine hormone and a local tissue paracrine and autocrine peptide in the kidney (14,20,22,27,30,41). ANG II, administered through the tubular lumen or via peritubular capillary perfusion, activates cell surface AT_1 receptors

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located in apical and basolateral membranes to regulate sodium and bicarbonate reabsorption under physiological conditions (14,22,41). Stimulating sodium and fluid reabsorption by ANG II in proximal tubules plays a major contributory role in sodium retention during the development of 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 intra-renal 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 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-AT_1 receptor complex is accompanied by increased phospholipase C- or phospholipase A_2-mediated sodium flux and decreased cAMP production in renal epithelial cells (4,29,30,34). These studies suggest that AT_{1A} 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 AT_1 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*) AT_1 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 AT_1 and AT_2 receptors), we demonstrated that AT_1 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 Val^5-ANG II, the radioligand [^{125}I]Tyr-ANG II, and ANG II enzyme immunoassay kits were obtained from Biochem/Peninsula Laboratories. cAMP enzyme immunoassay kits were purchased from R&D Systems. The AT_1 receptor antagonist losartan was a gift from Merck Pharmaceuticals, and the AT_2 receptor antagonist PD-123319 was donated by Pfizer. AT_1 receptor small-inteference RNA (siRNA) and rabbit polyclonal AT_1 receptor antibody targeting the NH_2-terminal extracellular domain of the human AT_1 receptor (N-10), scrambled siRNA, and transfection reagents were purchased from Santa Cruz Biotechnology. Western blot supplies were obtained from Amersham. Colchicine and phenylarsine oxide (PAO) were obtained from Calbiochem.

Cell culture

Unless specified otherwise, PTCs (*passages 8–12*) were subcultured in six-well plates in complete DMEM/F-12 growth medium supplemented with 50 nM hydrocortisone, 5% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (26,27,43). After reaching 80% confluence, they were starved in serum-free medium for 24 h before the experiment (26,27,43).

Expression of AT_1 and AT_2 receptors

To determine the proportion of AT_1 or AT_2 receptors in PTCs, we used [^{125}I]Tyr-ANG II binding assays as described previously (15,39,40). Briefly, the cells were incubated with [^{125}I]Tyr-ANG II (~100 pmol) for 60 min at 37°C. Total ANG II receptor binding was calculated as the binding in the absence of unlabeled ANG II or its receptor subtype-selective antagonist in the incubation. Nonspecific binding was determined as the binding in the presence of 10 μM unlabeled ANG II. AT_1 receptor binding was determined in the presence of 10 μM unlabeled AT_2 receptor blocker PD-123319, whereas AT_2 receptor binding was calculated as the binding in the presence of 10 μM unlabeled AT_1 blocker losartan. To determine ANG II receptor binding affinity constant (K_d) and maximum binding capacity (B_{max}), we produced saturation binding curves and Scatchard plot by incubating PTCs with increasing concentrations of [^{125}I]Tyr-ANG II (0–10 nM) alone or with 100 pmol of [^{125}I]Tyr-ANG II in the presence of increasing concentrations of unlabeled ANG II or losartan (0–10 μM). K_d and B_{max} were calculated using GraphPad Prism 4.0.

To confirm that rabbit PTCs express AT_1 receptor protein, we divided subconfluent (60%) cells into three groups ($n = 6$). The first group was treated with serum-free medium as a control. The second group was transfected with an AT_1 receptor-specific 20- to 25-nucleotide siRNA (AT_1R siRNA; Santa Cruz) (37). The third group was transfected with a negative, non-AT_1 receptor-targeting, scrambled siRNA (Santa Cruz). After transfection, cells were washed twice with ice-cold phosphate-uffered saline (PBS) and lysed with a modified RIPA buffer (50 mM Tris · HCl, 1% NP-40, 0.25% Nadeoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml each of aprotinin, leupeptin, and pepstatin, 1 mM Na_3VO_4, and 1 mM NaF, pH 7.4). Proteins were extracted, electrophoretically

separated on 8–16% Tris-glycine gels, and transferred to Millipore Immobilon-P membranes. The membranes were incubated for 3 h at room temperature with a rabbit polyclonal antibody against the human AT_1 receptor (N-10, 1:200; Santa Cruz) or a rabbit anti-AT_{1A} receptor polyclonal antibody against the cytosolic domain of the AT_{1A} receptor (CLSTKMSTLSYRPSDNM; 1:200) as described (11,17,42,43). 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).

AT_1 receptor-mediated endocytosis of extracellular ANG II

To determine whether AT_1 receptors are internalized by PTCs when exposed to extracellular ANG II, the cells were incubated with 100 pmol [^{125}I]Tyr-ANG II for 2, 5, 10, 15, or 30 min at 37°C alone or in the presence of the AT_1 receptor blocker losartan (10 μM) or the specific tyrosine phosphatase inhibitor PAO (1 μM), both known to inhibit AT_{1A} 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 AT_1 and AT_2 receptor blockade on intracellular accumulation of ANG II

To determine the role(s) of AT_1 receptor-mediated ANG II endocytosis, PTCs were treated with vehicle (serum-free medium), ANG II (Val^5-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, 10 mM EDTA, 5 mM EGTA, 5 mM mercaptoethanol, 50 g/ml 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, or tyrosine phosphatases in AT_1 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 cytoskeleton microtubule inhibitor colchicine (1 μM) (3,8,29), or ANG II plus the tyrosine phosphatase inhibitor PAO (1 μM) to block AT_1 receptor-mediated endocytosis (9,12,30). PAO is an established tyrosine phosphatase inhibitor that has been widely used for studying G protein-coupled receptor (GPCR) endocytosis (9,12,30,33,36). After treatment, the cells were washed and ANG II was extracted as described above.

Effects of AT_1 receptor-mediated endocytosis of extracellular ANG II on intracellular cAMP production

Cyclic AMP is one of the most important signaling molecules involved in the regulation of sodium and fluid transport by ANG II in proximal tubules (14,22,27,34). ANG II is thought to activate mainly basolateral AT_1 receptors, which are coupled to adenylyl cyclase via G_i proteins, to inhibit formation of cAMP; however, it was recently reported that after endocytosis, ANG II may directly activate G_i protein-coupled basolateral AT_1 receptors

(34). To determine whether AT_1 receptor-mediated ANG II endocytosis can affect intracellular cAMP signaling, we followed three experimental protocols. First, subconfluent PTCs in six-well plates were treated for 15, 30, or 60 min with serum-free medium only or ANG II (1 nM) alone to determine the time-dependent responses of cAMP production to ANG II (n = 6 each). Second, based on the time-dependent responses of cAMP to ANG II (peaked at 30 min), PTCs were pretreated with the AT_1 receptor blocker losartan (10 μM) or the tyrosine phosphatase inhibitor PAO (1 μM) before exposure to ANG II for 30 min (n = 6 each). Cells treated with losartan or PAO alone also were used as controls. Third, PTCs were treated for 30 min with *1*) the adenylyl cyclase activator forskolin alone (10 μM), which stimulates cAMP production, *2*) forskolin plus ANG II (ANG II + forskolin), *3*) ANG II plus forskolin and the AT_1 receptor blocker losartan (10 μM), *4*) ANG II plus forskolin and the AT_2 receptor blocker PD-123319 (10 μM), or *5*) ANG II plus forskolin and the endocytotic inhibitor PAO (1 μM) (n = 6 each). The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 1 mM) was added to all samples to prevent degradation of cAMP (29,33). After treatment, the culture medium was removed and the cells were washed and lysed with 0.1 N HCl. The lysates were centrifuged at 1000 *g*, and the supernatant was collected for cAMP measurements with the use of a sensitive cAMP ELISA kit (R&D).

Statistical analysis

Results are expressed as means ± SE. Unless otherwise specified, 6–12 samples from two separate experiments were collected for each treatment and assayed in duplicate for measurements of intracellular ANG II levels. For Western blot data, at least six samples from two separate experiments were performed, with each treatment assayed in duplicate. For binding data, two separate experiments were performed, with each time point determined in duplicate. Comparisons between two treatments were made using Student's unpaired *t*-test. Comparisons between more than two treatments were made with one-way analysis of variance, followed by a Newman-Keuls test for multiple comparisons. $P < 0.05$ was considered significant.

RESULTS

Expression of AT_1 receptors in rabbit proximal tubule cells

As shown in Fig. 1*A*, radioreceptor binding assays using [^{125}I]Tyr-ANG II showed that PTCs express a single class of ANG II receptor binding. Scatchard analysis suggests that these cells bound [^{125}I]Tyr-ANG II with an apparent K_d of 6.8 ± 2.1 nM, and the B_{max} averaged 1,012 ± 28 fmol/mg protein. The AT_1 receptor predominates in these cells because losartan (10 μM), a competitive AT_1 receptor blocker, displaced over 90% of total ANG II receptor binding, whereas the AT_2 receptor-selective blocker PD-123319 (10 μM) inhibited < 10% of binding. Next, we used two approaches to confirm that PTCs express AT_1 receptors. First, with the use of a rabbit anti-AT_1 receptor polyclonal antibody against the NH_2-terminal extracellular domain of the human AT_1 receptor (11,17,42), Western blot detected a single protein band of ~42 kDa (Fig. 1*B*). The AT_1 receptor protein we detected is consistent with the AT_1 receptor reportedly expressed in the rat kidney (15). In previous studies, pretreatment of samples with an AT_1 receptor-selective antigen blocking peptide (Santa Cruz) before running the Western blot confirmed the specificity of the AT_1 receptor protein (15,43). Second, AT_1 receptor protein expression was significantly knocked down by ~80% after transfection of PTCs with an AT_1-selective siRNA, whereas a scrambled, non-AT_1-selective siRNA had little effect (Fig. 1*B*). When the same membranes were stripped and reprobed with an anti-β-actin antibody, equal protein loading was confirmed (Fig. 1*A*, *bottom* band). Thus the PTCs we used express AT_1 receptor protein corresponding to human AT_1 receptors.

AT_1 receptor-mediated endocytosis of extracellular ANG II

AT_{1A} 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 AT_{1A} receptors because they do not express endogenous receptors (16,34). Using both the radioligand [^{125}I]Tyr-ANG II and fluorescein-conjugated ANG II, we determined whether extracellular ANG II is internalized after binding to cell surface AT_1 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 AT_1 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 AT_1 and AT_2 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 AT_1 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 Val^5-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 Val^5-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 AT_1 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, coadministration 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 AT_1 and AT_2 receptors mediate endocytosis of extracellular ANG II in PTCs, but it is AT_1 that predominated.

Effects of inhibitors of clathrin-coated pits, cytoskeleton microtubules, and tyrosine phosphatase on ANG II accumulation

Clathrin-coated pits, cytoskeleton microtubules, and tyrosine phosphatases have been separately shown to play animportant role in AT_{1A} receptor-mediated endocytosis in vascular smooth muscle cells (VSMCs) (2), Chinese hamster ovary (CHO) cells transfected with AT_{1A} receptors (9), or rat PTCs (29,30). However, it is not clear whether AT_1-mediated endocytosis contributes to accumulation of extracellular ANG II in rabbit PTCs via a similar mechanism. Figure 5*A* shows that incubation of PTCs at 4°C with Val^5-ANG II, which inhibits receptor-mediated endocytosis, prevented the AT_1-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. 5*B*, 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 AT_1 receptor-mediated endocytosis of extracellular ANG II on intracellular cAMP production

Previous studies suggested that AT_1 (or AT_{1A}) 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 AT_1-mediated endocytosis of extracellular ANG II has a functional relevance, we measured cAMP production in PTCs using an enzyme-linked immunoassay (R&D). AT_1-mediated ANG II endocytosis was blocked by the AT_1 receptor blocker losartan (10 μM) or the tyrosine phosphatase inhibitor PAO (1 μM). Figure 6*A* 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. 6*B*). 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 AT_1-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. 7*B*). 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 + forskolin) (Fig. 7*A*). These results indicate that AT_1 receptor-mediated endocytosis 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 AT_1 receptor protein equivalent to AT_{1A} receptors in rodents; *2*) incubating these cells with Val^5-ANG II significantly increased intracellular ANG II accumulation, which was blocked by inhibition of receptor-mediated endocytosis with the AT_1 receptor blocker losartan, cold (4°C), the cytoskeleton microtubule inhibitor colchicine, or the tyrosine phosphatase inhibitor PAO; and *3*) AT_1 receptor-mediated endocytosis of extracellular ANG II has a functional role, as indicated by the finding that blockade of AT_1 receptor-mediated endocytosis with various endocytotic inhibitors prevented ANG II-induced inhibition of basal and forskolin-stimulated intracellular cAMP production. These results suggest that AT_1-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 ANG II receptor-activated signaling. Because colchicine, a selective inhibitor of cytoskeleton microtubules, and PAO, an inhibitor of tyrosine phosphatases, prevented AT_1-mediated intracellular ANG II accumulation, our results suggest that increased endocytosis of extracellular ANG II in PTCs is cytoskeleton microtubule- and tyrosine phosphate-dependent. By contrast, hyperosmotic 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 AT_1 receptor-mediated ANG II accumulation in rabbit PTCs.

The present study provides evidence that AT_1-mediated ANG II endocytosis plays an important role in high intracellular accumulation of ANG II in PTCs in vitro and, by implication, that this process also may 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 AT_1-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 ^{125}I-labeled ANG II into pigs and measured labeled peptide levels in different cellular fractions of the kidney homogenates, they found that most ^{125}I-ANG II is cell-associated due to AT_1 receptor-mediated endocytosis. We previously measured internalized AT_{1A} receptors and ANG II in isolated renal cortical endosomes and intermicrovillar clefts of ANG II-infused rats and found that AT_{1A} 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 AT_1 receptor blocker candesartan prevented accumulation of extracellular ANG II in endosomes and intermicrovillar clefts, we interpreted these findings as an AT_{1A}-mediated response. However, it should be emphasized that all previous studies 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 AT_1-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 AT_1-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 AT_1 receptors. In the present study, we first demonstrated that AT_1 (equivalent to human AT_1 and rodent AT_{1A}) receptor proteins are expressed in these PTCs by Western blot, using rabbit anti-AT_1 receptor antibodies raised against the NH_2-terminal or cytosolic domains of the receptor (11,17,42) and an AT_1 receptor-selective siRNA. We then confirmed that incubating these cells with ^{125}I-ANG II induced ~80% internalization of the peptide within 30 min of exposure and that this was blocked by losartan, pointing to an AT_1 receptor-mediated mechanism. This phenomenon has been demonstrated previously in OK cells transfected with AT_{1A} 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 AT_1-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 AT_1-mediated endocytosis.

Whether AT_2 receptors mediate ANG II endocytosis and its subsequent intracellular signaling is not known (7,9,16,45). Hein et al. (16) showed that unlike AT_1 receptors, AT_2 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 AT_2 receptors and that the AT_2 receptor antagonist PD-123319 partially inhibited intracellular accumulation of extracellular ANG II, suggesting that endogenous AT_2 receptors perhaps play a minor role in mediating ANG II endocytosis in rabbit PTCs. Indeed, AT_2 receptors have been reported to mediate different biological effects in various renal cells (7).

The cellular mechanisms that regulate AT_1 (or AT_{1A}) 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 AT_{1A} 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 β_2-adrenergic receptors (9) and also with AT_{1A} 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 AT_1-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 AT_{1A} receptors (42); yet we could not determine whether clathrin-coated pits play any role in intracellular trafficking of ANG II/AT_1 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 (IP_3) signaling and ^{22}Na transport. In the present study, we demonstrated that colchicine and PAO completely prevented AT_1-mediated intracellular ANG II accumulation in PTCs, supporting the hypothesis that AT_1-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 AT_1-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 intracellular ANG II accumulation by inhibiting cytoplasmic trafficking of the peptide after endocytosis. In VSMCs, disruption of cytoskeleton microtubules with nocodazole blocked AT_1 receptor trafficking into caveolae/lipid rafts (45). By contrast, PAO may block AT_{1A}-mediated ANG II endocytosis via a different mechanism. PAO is a general inhibitor of tyrosine phosphatases that has been widely used to study AT_{1A} receptor endocytosis (12,30), but it is not clear which specific tyrosine phosphatase it inhibits and how it inhibits AT_{1A} receptor endocytosis. Previous studies have shown that PAO inhibits not only AT_{1A} 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 endocytotic 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_{1A} 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_{1A} receptor endocytosis and elucidate the cellular mechanisms involved.

Our results show that AT_1-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_1 receptor-mediated endocytosis was associated with decreased basal and forskolin-stimulated intracellular cAMP production. Losartan and PAO inhibited AT_1-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. 6*B*), other mechanisms unrelated to AT_1-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_1 receptor complex activated PLC-IP_3 signaling, increased sodium flux, and decreased cAMP signaling in cultured rat PTCs. Becker et al. (4) showed that AT_1 receptor-mediated endocytosis was associated with increased phospholipase A_2 activity and sodium flux in LLC-PK cells expressing rabbit AT_1 receptors. Thekkumkara and Linas (34) reported that in OK cells, apical membrane AT_{1A} receptors were internalized before they inter-act with G proteins, leading to inhibition of cAMP signaling. Accordingly, our finding that inhibition of AT_1 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_1 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_1 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_1

receptor-mediated intracellular ANG II accumulation blocks ANG II-inhibited cAMP production. These results suggest that AT_1 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.

Acknowledgments

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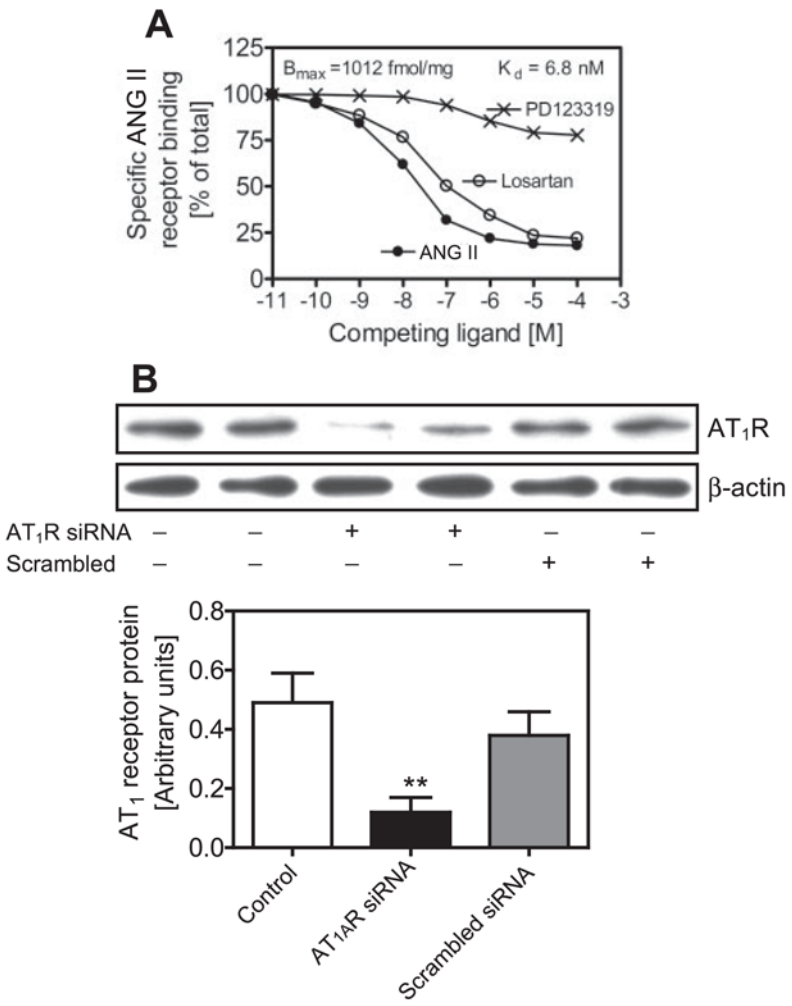

Fig. 1.

Expression of AT_1 receptors (equivalent to human AT_1 or rodent AT_{1A}) in cultured rabbit proximal tubule cells (PTCs). *A*: [^{125}I]Tyr-ANG II receptor binding showing binding competition by the unlabeled ANG II, AT_1 receptor blocker losartan or the AT_2 receptor blocker PD-123319 (10^{-11} to 10^{-4} M) and Scatchard analysis of maximum binding capacity (B_{max}) and the apparent K_d. *B*: ANG II receptor protein expression was significantly inhibited by a specific AT_1 receptor small-interference RNA (AT_1R siRNA), whereas a scrambled siRNA had no effect. $^{**}P < 0.01$ vs. control.

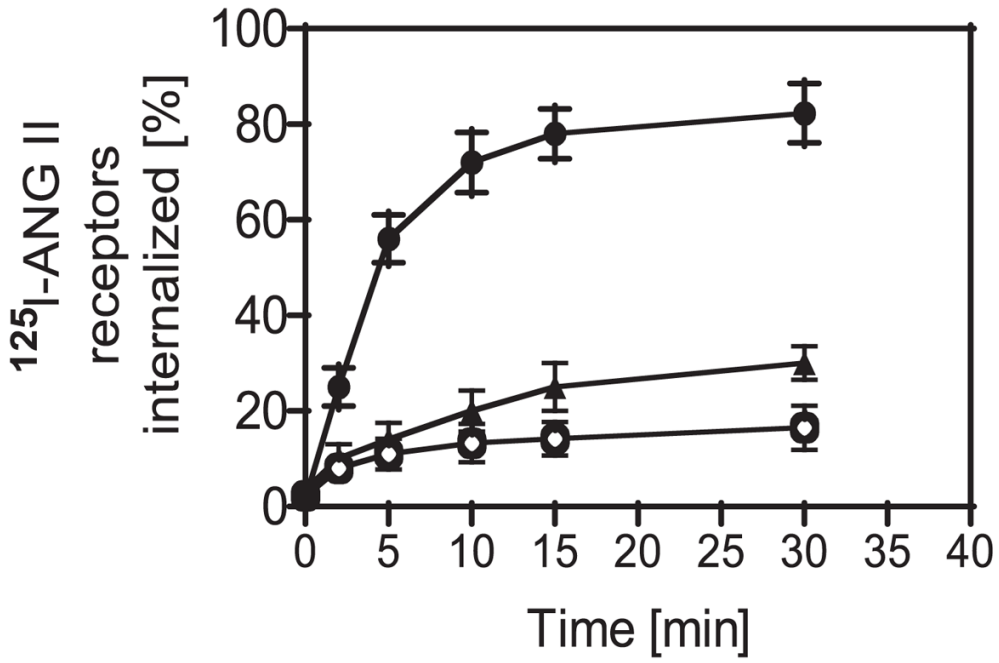

Fig. 2.

Time-dependent endocytosis of [^{125}I]Tyr-ANG II in cultured rabbit PTCs in the absence (●) or presence of the endocytotic inhibitor losartan (10 μM; ○) or phenylarsine oxide (PAO; 1 μM; ▲). Endocytosis of [^{125}I]Tyr-ANG II peaked at 30 min after exposure to the radioligand, which was significantly inhibited by losartan and PAO, suggesting an AT_1 receptor-mediated response.

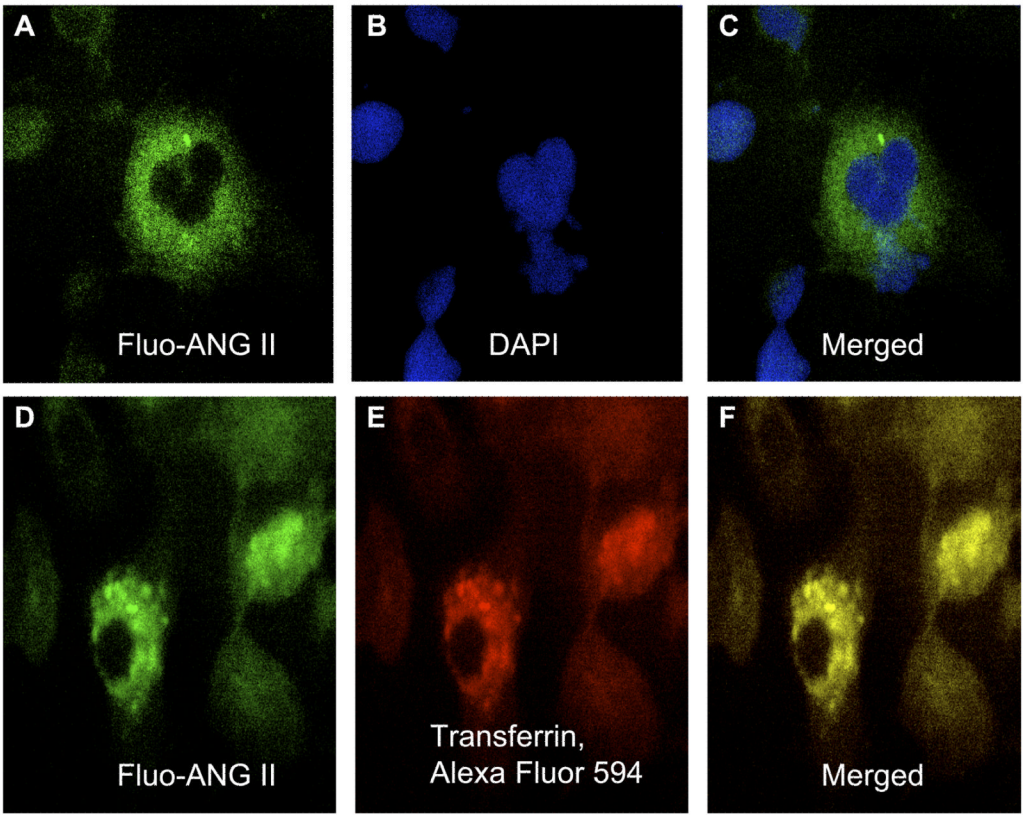

Fig. 3.

Endocytosis of fluorescein-labeled ANG II (Fluo-ANG II) in the cytoplasm of cultured rabbit PTCs, where it colocalized with Alexa Fluor 594-labeled transferrin, an endosomal marker, 30 min after the cells were exposed to Fluo-ANG II. *A*: cytoplasmic localization of internalized Fluo-ANG II (green). *B*: nucleus stained with 4,6-diamidino-2-phenylindole (DAPI; blue). *C*: merged image of *A* and *B*, showing the relationship between Fluo-ANG II and the nucleus. *D*: Fluo-ANG II (green). *E*: endosomes stained with Alexa Fluor 594-labeled transferrin (red). *F*: merged image of *D* and *E* (yellow), showing colocalization of Fluo-ANG II and transferrin. Magnification, ×40.

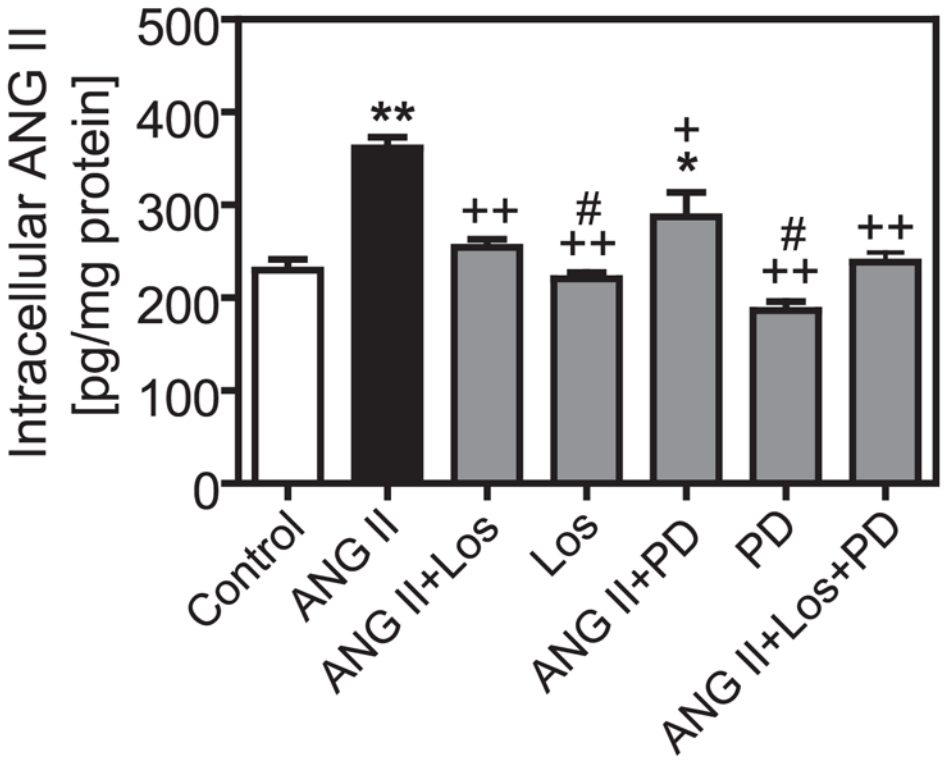

Fig. 4.

Effects of AT_1 and AT_2 receptors on intracellular accumulation of extracellular ANG II in PTCs. Note that both the AT_1 receptor blocker losartan (10 3M) and the AT_2 receptor blocker PD-123319 (10 μM) inhibited intracellular ANG II accumulation, but the effect of losartan predominated. Los, losartan; PD, PD-123319. **P* < 0.05; ***P* < 0.01 vs. control. + *P* < 0.05; + + *P* < 0.01 vs. ANG II. #*P* < 0.05 vs. ANG II + Los or ANG II + PD.

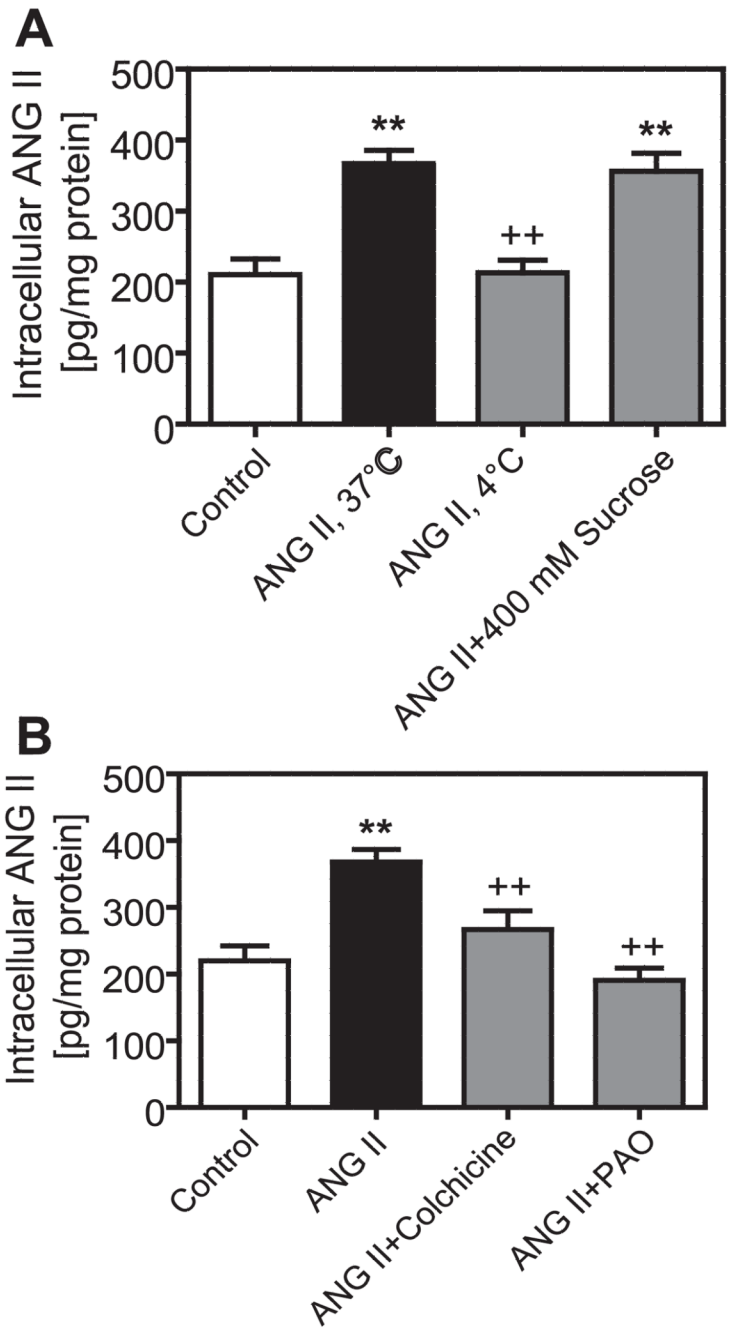

Fig. 5.

Effects of endocytotic inhibitors on AT_1 receptor-mediated accumulation of ANG II in PTCs. *A*: effects of cold (4°C), which inhibits AT_1 receptor-mediated endocytosis, or sucrose (400 mM), which depletes clathrin-coated pits in non-renal cells. *B*: effects of the cytoskeleton microtubule inhibitor colchicine (1 μM) or the tyrosine phosphatase inhibitor PAO (1 μM). $^{**}P < 0.01$ vs. control. + + $P < 0.01$ vs. ANG II.

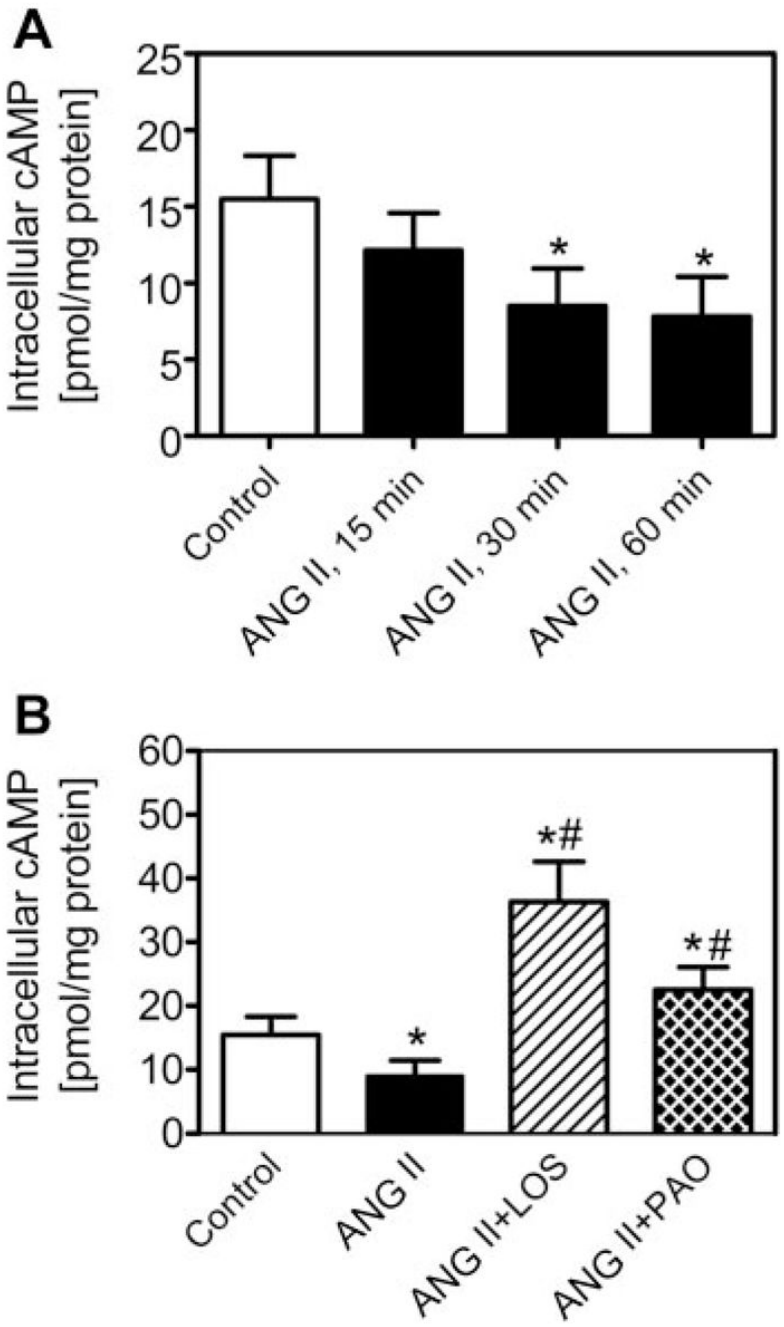

Fig. 6.

Effects of blockade of AT_1-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.

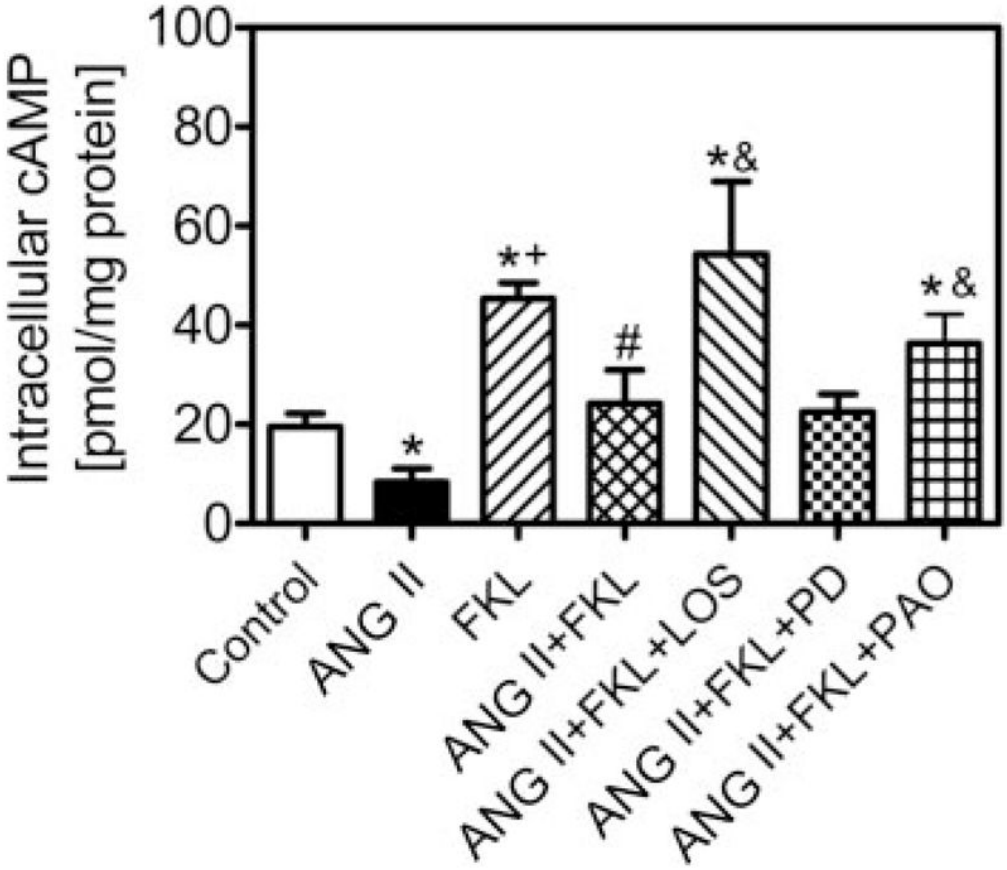

Fig. 7.

Effects of blockade of AT_1-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.