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Intracellular ANG II induces cytosolic Ca^{2+} mobilization by stimulating intracellular AT_1 receptors in proximal tubule cells

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Abstract

Intracellular ANG II induces biological effects in nonrenal cells, but it is not known whether it plays a physiological role in renal proximal tubule cells (PTCs). PTCs express angiotensinogen, renin, and angiotensin-converting enzyme mRNAs, suggesting the presence of high levels of intracellular ANG II. We determined if microinjection of ANG II directly in single PTCs increases intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) and, if so, elucidated the cellular mechanisms involved. Changes in $[\text{Ca}^{2+}]_i$ responses were studied by fluorescence imaging using the Ca^{2+} indicator fluo 3. ANG II (1 nM) was microinjected directly in the cells, whereas cell-surface angiotensin type 1 (AT_1) receptors were blocked by losartan (10 μM). When ANG II (1 nM) was added to the perfusate, there was a marked increase in $[\text{Ca}^{2+}]_i$ that was blocked by extracellular losartan. With losartan in the perfusate, intracellular microinjection of ANG II elicited a robust increase in cytoplasmic $[\text{Ca}^{2+}]_i$ that peaked at 30 s (basal: 2.2 ± 0.3 vs. ANG II: 14.9 ± 0.4 relative fluorescence units; $P < 0.01$). Chelation of extracellular Ca^{2+} with EGTA (2 mM) did not alter microinjected ANG II-induced $[\text{Ca}^{2+}]_i$ responses (Ca^{2+} free + ANG II: 12.3 ± 2.6 relative fluorescence units, not significant vs. ANG II); however, pretreatment with thapsigargin to deplete intracellular Ca^{2+} stores or with U-73122 to inhibit phospholipase C (1 μM each) markedly attenuated microinjected ANG II-induced $[\text{Ca}^{2+}]_i$ responses. Combined microinjection of ANG II and losartan abolished $[\text{Ca}^{2+}]_i$ responses, whereas a combination of ANG II and PD-123319 had no effect. These data demonstrate for the first time that direct microinjection of ANG II in single PTCs increases $[\text{Ca}^{2+}]_i$ by stimulating intracellular AT_1 receptors and releases Ca^{2+} from intracellular stores, suggesting that intracellular ANG II may play a physiological role in PTC function.

Keywords

intracellular calcium; kidney; microinjection; proximal tubules; receptor-mediated endocytosis

ANG II is a Powerful peptide hormone that exerts both endocrine and paracrine actions in the kidney (3,11,16,20,35). Physiologically, ANG II maintains body sodium and fluid balance and blood pressure homeostasis by stimulating tubular sodium and fluid reabsorption as well as through its direct effects on the vasculature (11,20,33,38). However, sustained increases in intrarenal ANG II levels because of local formation and/or uptake of circulating and locally formed extracellular ANG II by proximal tubule cells (PTCs) may contribute to sodium retention and promote cell growth and proliferation, leading to hypertension-mediated renal injury (19,21,36). It is now recognized that PTCs express all

necessary components of the rennin-angiotensin system (RAS), including the precursor angiotensinogen as well as renin and angiotensin-converting enzyme (ACE; see Refs. 12,14,16,28). Moreover, ANG II concentrations in proximal tubular fluid and renal cortical interstitial fluid are greater than can be explained by the circulating ANG II concentrations (2,22,27). These findings suggest that substantial amounts of ANG II may be generated within PTCs. However, there is also mounting evidence that binding of extracellular ANG II to membrane-bound angiotensin type 1 (AT_1) receptors promotes internalization of extracellular ANG II into various cells, including vascular smooth muscle cells (VSMCs; see Refs. 1 and 9) and renal epithelial cells (13,25,31). In vivo, ANG II has been shown to accumulate in the kidney after salt restriction (17) and chronic ANG II administration (33,36,40,41), where internalized extracellular ANG II was colocalized with AT_{1A} receptors in the endosomal compartments of the rat renal cortex (36). These studies suggest that, after internalization, ANG II may also exert an important intracrine role in regulating PTC function.

Although it is widely accepted that ANG II exerts diverse effects by activating cell-surface AT_1 receptors in targeted cells (6,39), recent studies suggest that intracellular ANG II, either accumulated through internalization or synthesized within the cells, is also capable of inducing intracellular responses (5,7–10). Haller et al. (10) demonstrated that microinjection of ANG II in VSMCs increased intracellular $[Ca^{2+}]_i$, and this response was blocked by intracellular administration of candesartan, an AT_1 receptor antagonist (10). Similarly, dialysis of ANG II in VSMCs or cardiac myocytes has been shown to stimulate voltage-operated Ca^{2+} channels (8) or reduce inward calcium currents (7). Cook et al. (5) recently reported that ANG II generated within hepatocytes appeared to exert mitogenic effects. Although PTCs are known to produce and accumulate high levels of ANG II, it remains unclear whether intracellular ANG II exerts any role in regulating proximal tubular cell function. PTCs are structurally and functionally different from VSMCs, hepatocytes, or cardiac myocytes, and they may have completely different responses to extracellular and intracellular ANG II. Studies by Schelling and Linas (25) suggested that endocytosis of the ANG II- AT_1 receptor complex may be required for full expression of biological actions of ANG II in PTCs. Moreover, Thekkumkara et al. (31,32) demonstrated that internalization of an AT_{1A} receptor-ANG II complex increases transcellular sodium transport in opossum kidney cells. Although these results suggest possible roles for intracellular ANG II, the influence of intracellular ANG II in PTC function remains poorly understood. In the present study, we hypothesized that intracellular ANG II activates cytoplasmic AT_1 receptors to increase intracellular calcium concentration ($[Ca^{2+}]_i$) by mobilizing Ca^{2+} from intracellular stores in PTCs. We found that microinjection of ANG II directly into single PTCs induced intracellular Ca^{2+} responses that were mediated by intracellular AT_1 receptors and mobilization of intracellular Ca^{2+} stores but not dependent on influx of extracellular Ca^{2+} .

MATERIALS AND METHODS

PTC culture

Cultured PTCs were obtained from American Type Culture Collection and subcultured as described previously (16,24,28). These cells were initially derived from the S_1 segment of rabbit kidney proximal tubules and have been shown to express electrolyte transporters and major components of the RAS, including angiotensinogen, renin, ACE, and ANG II receptors (24). In the present study, unless specified otherwise, PTCs were subcultured on glass coverslips for measurements of $[Ca^{2+}]_i$ responses in complete DMEM-F-12 growth medium supplemented with 50 nM hydrocortisone, 5% heat-inactivated FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cells were maintained at 37°C and 95% O_2 -5% CO_2 and fed every 2–3 days. Serum was removed from the medium for 24 h before the experiments began (16,28).

Expression of AT₁ receptor protein in PTCs

To confirm that PTCs we used express AT₁ receptors, the cells were split into six-well plates and grown to 80% confluence as described above. After being serum starved for 24 h, cells were washed two times with ice-cold PBS and lysed with a modified RIPA buffer [50 mM Tris · HCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml each of aprotinin, leupeptin, and pepstatin, 1 mM Na₃VO₄, and 1 mM NaF, pH 7.4]. Samples were extracted as described previously (12), and protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce). Proximal tubule protein samples (10 µg each) were electrophoretically separated on 8–16% Tris-glycine gels at 120 volts for 1.5–2 h. After SDS separation, proteins were transferred to Millipore Immobilon-P membranes using a Bio-Rad Trans-Blot Semi-Dry system powered by a Bio-Rad Power-Pac HC (25 volts, 0.12 ampere, 1.5 h). The membranes were blotted overnight at 4°C with 5% nonfat dry milk and incubated for 3 h at room temperature with a primary rabbit anti-AT₁ receptor polyclonal antibody raised against the NH₂-terminal extracellular domain of AT₁ receptors (1:200, SC-1173; Santa Cruz; see Ref. 12). To determine the specificity of the antibody, an AT₁ receptor-selective blocking peptide was used to treat the samples before running the Western blots (SC-1173BP; Santa Cruz). To ensure equal protein loading, the same membranes were treated with a stripping buffer (Pierce) for 20 min, blotted with 5% nonfat dry milk, and reprobed with a mouse anti-β-actin monoclonal antibody at 1:2,000 (Sigma-Aldrich). Western blot signals were detected using enhanced chemiluminescence (Amersham) and analyzed using a microcomputer imaging device with a digital camera (MCID; Imaging Research).

Measurement of intracellular ANG II in PTCs

To confirm that PTCs we used produce endogenous ANG II and exposure of these cells to extracellular ANG II increases intracellular ANG II levels via AT₁ receptor-mediated endocytosis, cells were treated with vehicle (serum-free medium), ANG II (1 nM), or ANG II plus losartan (10 µM) to block receptor-mediated endocytosis for 60 min at 37°C. After treatment, the medium was removed, and the cells were washed two times with ice-cold PBS and then washed one time with ice-cold acid buffer (5 mM acetic acid, 150 mM NaCl, pH 2.5) to remove any remaining cell membrane-bound ANG II as described previously (1,9,13,32). 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 and measured using a sensitive and specific ANG II enzyme immunoassay kit (Biochem/Peninsula). Protein concentration in each sample was used to calculate final ANG II levels expressed as picograms per milligram protein.

Monitoring [Ca²⁺]_i responses in single PTCs

Subconfluent PTCs were sparsely plated on glass coverslips and loaded with the calcium-sensitive fluorescent dye fluo 3-AM (2 µM; Molecular Probes), which was prepared in PBS for 30 min at 37°C (10,18). After incubation, coverslips with attached cells were rinsed two times with PBS to remove extracellular dye and extracellular agonist(s) and placed inside a perfusion chamber mounted on a Nikon Diaphot 200 inverted microscope with a TE-FM epifluorescence attachment and a fluorescence filter suitable for fluo 3 (excitation: 488-nm wavelength; emission: 526; see Ref. 10). The cells were continuously maintained in the perfusate (10 mM HEPES, 1.5 mM CaCl₂, 145 mM NaCl, 5 mM KCl, 0.5 Na₂HPO₄, 6 mM glucose, and 0.5 mM MgSO₄, pH 7.4) at 37°C. The [Ca²⁺]_i responses to extracellular and intracellular ANG II were recorded with a digital camera and microcomputer imaging device (Imaging Research), first at basal levels and then at 5-s intervals after microinjection of ANG II until responses returned to baseline.

Effect of extracellular ANG II and activation of cell-surface AT₁ receptors on [Ca²⁺]_i in PTCs

Experiments were first carried out to confirm that extracellular ANG II increases [Ca²⁺]_i in PTCs and that these effects are blocked when losartan is added to the perfusate. ANG II was added to the perfusate at 1 nM and losartan at 10 μM. These concentrations were based on the observations that proximal tubule fluid and interstitial fluid contain nanomolar levels of ANG II (2,22,27) and that at 10 μM losartan completely displaced ANG II receptor binding in rat proximal tubules (36–38).

Effect of intracellular microinjection of ANG II on [Ca²⁺]_i in single PTCs

To determine whether intracellular ANG II increases [Ca²⁺]_i, ANG II (1 nM; Bachem) was microinjected directly into single PTCs while blocking cell-surface AT₁ receptors with losartan in the perfusate (10 μM; Fig. 1). Intracellular microinjection of ANG II was carried out using a Narishige micromanipulator and microinjector at a volume of ~70–100 fL/cell with a glass micropipette (10). Success of intracellular microinjection of ANG II was confirmed using FITC-labeled ANG II (Molecular Probes) to follow intracellular trafficking of microinjected FITC-ANG II and to exclude possible leaking of microinjected ANG II in the extracellular space (Fig. 1).

Role of intracellular AT₁ or AT₂ receptors in [Ca²⁺]_i responses to microinjected ANG II in single PTCs

To determine whether intracellular effects of microinjected ANG II are mediated by intracellular AT₁ or AT₂ receptors, either the AT₁ receptor blocker losartan or the AT₂ receptor blocker PD-12319 was microinjected in the cells together with ANG II at a final concentration of 10 μM in the injection to block intracellular AT₁ or AT₂ receptors. [Ca²⁺]_i responses in a single cell on a cover slip were recorded continuously for up to 10 min after microinjection.

Role of extracellular Ca²⁺ in [Ca²⁺]_i responses to microinjected ANG II in single PTCs

To determine whether the effects of microinjected ANG II on [Ca²⁺]_i are dependent on influx of extracellular Ca²⁺, cells were perfused in a medium containing zero Ca²⁺ with 2 mM EGTA added while ANG II was injected into the cells (10). [Ca²⁺]_i responses in a single cell on a coverslip were recorded for up to 10 min after ANG II microinjection.

Role of intracellular calcium stores in [Ca²⁺]_i responses to microinjected ANG II in single PTCs

Cells were first pretreated with 1 μM thapsigargin for 30 min to deplete intracellular calcium stores before microinjecting ANG II in the cells (10,18). [Ca²⁺]_i responses in a single cell on a cover slip were recorded for up to 10 min after microinjection.

Role of phospholipase C/protein kinase C signaling pathway(s) in [Ca²⁺]_i responses to microinjected ANG II in single PTCs

Cells were pretreated with U-73122 (1 μM), a selective phospholipase C (PLC) inhibitor, for 30 min before microinjecting ANG II in the cells. [Ca²⁺]_i responses in a single cell on a cover slip were recorded for up to 10 min after microinjection.

Statistical analysis

Data are presented as means ± SE. [Ca²⁺]_i responses as determined by fluo 3 fluorescence imaging, represented as relative fluorescence units. Differences in intracellular ANG II levels or [Ca²⁺]_i responses between different groups of cells were analyzed using one-way

ANOVA followed by Dunnett's comparison between group means, taking $P < 0.05$ as significant.

RESULTS

Western blot of AT₁ receptor protein in PTCs

Western blot showed a single protein band of ~42 kDa (Fig. 2A, *top*) in PTCs, which was markedly inhibited by an AT₁ receptor-selective blocking peptide (Fig. 2, *A and B*). The AT₁ receptor protein detected is consistent with the AT₁ receptor reportedly expressed in the rat kidney (12). When the same membranes were stripped and reprobed with an anti- β -actin antibody, equal protein loading was confirmed (Fig. 2A, *bottom*). Thus these results confirm that PTCs used in the present study express specific AT₁ receptor protein.

Intracellular ANG II levels in PTCs

Figure 3 shows intracellular ANG II levels under basal conditions, during incubation with ANG II (1 nM) to promote receptor-mediated endocytosis, and in the presence of losartan to assess the role of AT₁ receptors on ANG II endocytosis. Basal ANG II levels in PTCs averaged 97.6 ± 5.0 pg/mg protein. Incubation with 1 nM ANG II significantly increased intracellular ANG II levels by $47.4 \pm 6.0\%$ (143.9 ± 6.0 pg/mg protein, $P < 0.001$). Losartan completely prevented receptor-mediated increases in ANG II concentrations in PTCs [90.1 ± 3.0 pg/mg protein, not significant (NS)].

Effects of extracellular ANG II on $[Ca^{2+}]_i$ responses

Before PTCs were exposed to extracellular ANG II, there were minimal levels of basal Ca^{2+} fluorescence (2.8 ± 0.6 relative fluorescence units, $n = 24$; Fig. 4A). Adding ANG II (1 nM) to the perfusate elicited a rapid and sustained increase in $[Ca^{2+}]_i$ in all cells (14.3 ± 0.3 relative fluorescence units, $n = 24$; Fig. 4B). The response peaked at 30 s and returned to basal levels 3–5 min later. In another group of cells ($n = 20$), prior administration of losartan (10 μ M) in the perfusate effectively abolished the $[Ca^{2+}]_i$ response to extracellular ANG II (3.6 ± 0.2 relative fluorescence units; Fig. 4C). Figure 4D shows relative calcium fluorescence levels in three different groups of cells. These data confirmed that addition of losartan (10 μ M) in the perfusate effectively blocked $[Ca^{2+}]_i$ signaling induced by extracellular ANG II.

Effects of intracellular microinjection of ANG II on intracellular Ca^{2+} mobilization

To determine the effects of intracellular ANG II on $[Ca^{2+}]_i$ responses, ANG II was microinjected directly in single PTCs. Because losartan was applied to the perfusate to block cell-surface AT₁ receptors, this approach separates the effects of ANG II on $[Ca^{2+}]_i$ responses mediated by cell-surface receptors from those mediated by intracellular receptors. Figure 5, A–E, shows the time dependency of the changes of $[Ca^{2+}]_i$ (0, 15, 30, 60, and 120 s) in a representative PTC after ANG II was microinjected directly in the cytoplasm, and Fig. 5F shows the quantitated results ($n = 12$ cells). Intracellular microinjection of ANG II induced a rapid increase, as indicated by relative fluorescence intensity, in $[Ca^{2+}]_i$ signaling throughout the cells, including the nucleus, which peaked at 30 s (basal: 2.2 ± 0.3 fluorescence units vs. ANG II: 14.9 ± 0.4 relative fluorescence units, $P < 0.01$). However, the fluorescence calcium imaging we used could not quantify the differences between nuclear and cytosolic responses. The $[Ca^{2+}]_i$ response returned to baseline levels ~3 min after microinjection (2.6 ± 0.3 relative fluorescence units). Similar calcium responses were observed after microinjection of FITC-ANG II ($n = 5$), which was trafficked through the cytoplasm (Fig. 1D). In a separate group of cells ($n = 6$), intracellular microinjection of a similar volume of the buffer used for ANG II microinjection did not induce a significant

$[Ca^{2+}]_i$ response (3.1 ± 0.6 relative fluorescence units). We did not observe significant increases in $[Ca^{2+}]_i$ signaling in adjacent cells because we plated PTCs sparsely on cover slips so that cells were not in close contact with each other to facilitate microinjection. However, in confluent monolayers of PTCs, microinjection of ANG II in a single cell also elicited $[Ca^{2+}]_i$ signaling in adjacent cells. Pretreatment of monolayer PTCs with U-73122 to inhibit PLC diminished $[Ca^{2+}]_i$ signaling in adjacent cells. Taken together, because we added losartan ($10 \mu M$) to the perfusate to block cell-surface AT_1 receptors, these data suggest that intracellular ANG II activates cytoplasmic AT_1 receptors to increase $[Ca^{2+}]_i$ in PTCs.

Effects of intracellular AT_1 and AT_2 receptors on $[Ca^{2+}]_i$ responses to microinjected ANG II

Because PTCs express both AT_1 and AT_2 receptors (16,37), we next examined the receptors responsible for the microinjected ANG II-induced $[Ca^{2+}]_i$ responses in single PTCs using subtype-selective antagonists. The AT_1 antagonist losartan ($10 \mu M$) or the AT_2 antagonist PD-123319 ($10 \mu M$) was microinjected together with ANG II, and $[Ca^{2+}]_i$ responses were recorded every 5 s for up to 10 min. Figure 6 shows peak $[Ca^{2+}]_i$ responses after microinjection of ANG II for 10 min ($n = 12$). The $[Ca^{2+}]_i$ response to ANG II reached a maximum between 15 and 30 s (14.2 ± 0.5 relative fluorescence units) and returned to baseline after 3 min (4.3 ± 0.3 relative fluorescence units). This response was inhibited by concurrent microinjection of losartan (5.8 ± 0.6 relative fluorescence units, $P < 0.01$ vs. ANG II alone; $n = 10$) but not by PD-123319 (13.1 ± 0.3 relative fluorescence units, NS vs. ANG II alone; $n = 8$), indicating that it is mediated by activation of intracellular AT_1 receptors.

Effect of extracellular Ca^{2+} on $[Ca^{2+}]_i$ responses to microinjected ANG II

Previous studies of VSMCs showed that intracellular ANG II increased $[Ca^{2+}]_i$ levels and stimulated voltage-operated Ca^{2+} channels via an extracellular Ca^{2+} -dependent mechanism (7,8). In the present study, we examined if increases in $[Ca^{2+}]_i$ induced by microinjected ANG II are altered by removing extracellular Ca^{2+} from the perfusate (Ca^{2+} -free medium + 2 mM EGTA). In these experiments, ANG II was microinjected at the same rate and concentration except that extracellular Ca^{2+} was completely removed. Figure 7, A–F, shows the time-dependent changes in $[Ca^{2+}]_i$ responses in a representative PTC, and Fig. 7G shows quantitated data from a group of cells ($n = 8$). Removal of extracellular Ca^{2+} did not alter the time course and magnitude of $[Ca^{2+}]_i$ responses to microinjected ANG II (12.3 ± 2.6 relative fluorescence units, NS vs. ANG II alone). These data suggest that intracellular ANG II may increase $[Ca^{2+}]_i$ primarily via release from intracellular stores, rather than influx of extracellular Ca^{2+} .

Effects of depletion of intracellular Ca^{2+} stores with thapsigargin on $[Ca^{2+}]_i$ responses induced by microinjected ANG II

Because removal of extracellular Ca^{2+} from the perfusate did not significantly alter $[Ca^{2+}]_i$ responses to microinjected ANG II, PTCs were first treated with $1 \mu M$ thapsigargin for 30 min to deplete intracellular calcium stores before microinjecting ANG II. Thapsigargin has been widely used to study the role of intracellular calcium mobilization in agonist-induced $[Ca^{2+}]_i$ signaling in cardiovascular and renal cells (10,15,18). Figure 8A shows that pretreatment of PTCs with thapsigargin completely abolished the increases in $[Ca^{2+}]_i$ induced by microinjected ANG II (3.6 ± 1.2 relative fluorescence units, NS vs. basal; $n = 6$).

Role of activation of PLC on $[Ca^{2+}]_i$ responses induced by microinjected ANG II in single PTCs

AT₁ receptor-activated PLC and resulting formation of inositol trisphosphate (IP₃) are the hallmark upstream signaling pathways by which extracellular ANG II induces $[Ca^{2+}]_i$ responses in various cells. It is not known if intracellular ANG II can activate this signaling pathway to induce $[Ca^{2+}]_i$ responses in single PTCs. Figure 8B shows that pretreatment of PTCs with U-73122 (1 μ M) to inhibit PLC markedly diminished microinjected ANG II-induced calcium signaling (control: 2.6 ± 0.3 relative fluorescence units; ANG II: 12.6 ± 0.6 relative fluorescence units, $P < 0.01$ vs. control; U-73122 + ANG II: 3.5 ± 0.5 relative fluorescence units, $P < 0.01$ vs. ANG II), indicating that PLC is involved in intracellular action of ANG II on $[Ca^{2+}]_i$ mobilization in PTCs.

DISCUSSION

The present study demonstrates for the first time that microinjection of ANG II directly in single PTCs increases $[Ca^{2+}]_i$ levels by activating intracellular AT₁ receptors. Because $[Ca^{2+}]_i$ responses to microinjected ANG II were elicited while extracellular losartan was applied to block cell-surface AT₁ receptors, the results indicate that the microinjected ANG II exerted an intracellular effect. Furthermore, because $[Ca^{2+}]_i$ responses to microinjected ANG II were abolished by coadministration of losartan but not PD-123319, and because removal of extracellular Ca^{2+} did not affect the intracellular actions of microinjected ANG II, our results suggest that intracellular AT₁ receptors, either internalized from cell membranes or synthesized within the cell, mediate the observed effects via an intracellular mechanism(s). Our results are consistent with previous observations that microinjection of ANG II in VSMCs increased $[Ca^{2+}]_i$ (10) and suggest that intracellular ANG II plays an important intracrine role in PTC function.

Re and associates (5,23) have supported the concept that intracellular ANG II may play an important intracrine role in hepatocytes and cardiovascular cells. Although intracellular ANG II has been shown to have significant effects in nonrenal cells, it remains unclear if it exerts any biological or physiological role in PTCs. Several lines of evidence suggest that intracellular ANG II may play a role in PTC function. Expression of all necessary components of the RAS in these cells strongly suggests such a possibility (2,14,16,28,36). Moreover, high levels of ANG II in proximal tubular fluid and interstitial fluid compartments may promote AT₁ receptor-mediated endocytosis of ANG II in PTCs, where it could induce intracellular responses (2,22,36). Imig et al. (14) demonstrated the presence of ANG I, ANG II, ACE, and AT_{1A} receptors in rat renal cortical endosomes; most were likely of the PTC origin. Further studies by us demonstrated increased accumulation of extracellular ANG II in endosomes and inter-microvillar clefts isolated from the renal cortex of rats chronically infused with ANG II (36). However, it has not been shown whether intracellular ANG II, either synthesized intracellularly or internalized from extracellular compartments, exerts a physiological effect. Schelling and Linas (25) demonstrated that, in isolated PTCs, phenylarsine oxide, which inhibits ANG II receptor internalization, also blocks ANG II-stimulated proximal tubule sodium transport. This suggests indirectly that ANG II-dependent proximal tubule sodium transport requires receptor-mediated endocytosis of ANG II. In the present study, we directly microinjected ANG II in single PTCs and monitored changes in $[Ca^{2+}]_i$ signaling. To ensure that $[Ca^{2+}]_i$ responses to microinjected ANG II were not mediated by cell-surface AT₁ receptors, we added losartan to the perfusate to block membrane AT₁ receptors while injecting ANG II in the cells, as described by Haller et al. (10). The increases in $[Ca^{2+}]_i$ induced by microinjected ANG II thus indicate that it was elicited as a consequence of the intracellular ANG II acting on an intracellular receptor.

The mechanisms by which intracellular ANG II induces $[Ca^{2+}]_i$ responses in PTCs are not fully understood. There are two important mechanisms that determine $[Ca^{2+}]_i$ levels within the cell (influx of extracellular Ca^{2+} and Ca^{2+} released from intracellular stores). In the present study, we observed that removal of extracellular Ca^{2+} did not alter the time course or the extent of increases in $[Ca^{2+}]_i$ responses to microinjected ANG II. Our results suggest that the $[Ca^{2+}]_i$ responses to microinjected ANG II do not depend significantly on influx of extracellular Ca^{2+} but are primarily because of Ca^{2+} release from intracellular stores in PTCs. These observations are different from those reported in VSMCs by Haller et al. (10) in that intracellular Ca^{2+} responses to microinjected ANG II were primarily the result of influx of extracellular Ca^{2+} in VSMCs. In that same study, treatment of the cells with EGTA, which removes Ca^{2+} from the medium, or with nitrendipine, an L-type Ca^{2+} channel antagonist, both markedly inhibited $[Ca^{2+}]_i$ responses in VSMCs microinjected with ANG II (10). In cardiac (7) or arterial (8) myocytes, dialysis of ANG II in the cells either stimulated or reduced Ca^{2+} channel activity, which may alter $[Ca^{2+}]_i$ levels in response to intracellular ANG II. Thus the effects of extracellular Ca^{2+} on the $[Ca^{2+}]_i$ responses to microinjected ANG II appear to be dependent on the cells studied. For example, depletion of intracellular Ca^{2+} stores with cyclopiazonic acid or thapsigargin markedly attenuated ANG II-induced constriction of pre- and postglomerular arterioles in the kidney (15,18).

The present study shows that, in PTCs, the $[Ca^{2+}]_i$ responses to microinjected ANG II are mediated primarily by activating AT_1 receptors inside PTCs. This conclusion is supported by previous observations that AT_1 receptors predominate in proximal tubules (36,37) and that concurrent microinjection of losartan and ANG II almost completely abolished $[Ca^{2+}]_i$ responses, whereas the AT_2 receptor blocker PD-123319 had little effect. The present results are consistent with the reported $[Ca^{2+}]_i$ or cell membrane voltage-operated Ca^{2+} channel response to intracellular ANG II that was abolished by candesartan in VSMCs (10) or arterial myocytes (8). However, they contrast with the effect of losartan in cardiac myocytes, where losartan did not block intracellular ANG II-induced changes in membrane calcium currents (7). The reason for the differences between cardiac myocytes and PTCs is not clear. It is well documented that AT_1 receptors are internalized in the endosomal compartments after exposure to extracellular ANG II in VSMCs (1,9) and renal epithelial cells (13,32,33). Moreover, AT_{1A} receptors have been shown to colocalize with ANG II in isolated rat renal cortical endosomes derived primarily from PTCs (14,36). Although it remains to be determined how intracellular ANG II interacts with the AT_1 receptor to induce $[Ca^{2+}]_i$ responses, our results suggest that intracellular AT_1 receptors are activated by intracellular ANG II to increase $[Ca^{2+}]_i$ through mobilization of intracellular Ca^{2+} stores.

Our results further show that activation of PLC may be involved in intracellular ANG II-induced $[Ca^{2+}]_i$ responses in PTCs. In many ANG II-targeted cells (for instance, VSMCs), biological effects of extracellular ANG II in PTCs are often mediated by activation of PLC, followed by the hydrolysis of phosphatidylinositol 4,5-bisphosphate to form IP_3 and diacylglycerol (6,9,26). Increases in IP_3 and diacylglycerol in turn induce intracellular $[Ca^{2+}]_i$ mobilization and activation of protein kinase C (9,26,34). Together with inhibition of adenylyl cyclase to decrease cAMP production, these intracellular signaling pathways play an important role in ANG II-regulated proximal sodium and fluid transport. In the present study, we found that intracellular ANG II-induced increases in $[Ca^{2+}]_i$ could be prevented by pretreating the cells with the PLC inhibitor U-73122. Interestingly, Schelling et al. (26) reported that inhibition of PLC with U-73122 decreased apical ANG II-induced sodium flux in isolated rat PTCs. It is therefore likely that activation of PLC is required for both extracellular and intracellular ANG II-induced calcium signaling and sodium transport in PTCs.

In summary, we have demonstrated for the first time that intracellular microinjection of ANG II in single PTCs increases $[Ca^{2+}]_i$ and that these responses do not depend significantly on influx of extracellular Ca^{2+} but instead require activation of intracellular AT_1 receptors and PLC. Our results suggest that intracellular ANG II, either derived from endocytosis of extracellular ANG II or synthesized within the cell, exerts an important intracrine role in $[Ca^{2+}]_i$ signaling in PTCs. However, it remains unclear how the increase in $[Ca^{2+}]_i$ in response to intracellular ANG II stimulation is related to the known effects of ANG II on proximal tubular sodium and fluid transport. Early studies by Taylor and Windhager (30) showed that marked elevation of $[Ca^{2+}]_i$ was associated with reduced transepithelial sodium transport. By contrast, small increases in $[Ca^{2+}]_i$ have been reported to stimulate sodium flux in cultured renal epithelial cells (30). Moreover, the complex intracellular mechanisms by which microinjected ANG II increases $[Ca^{2+}]_i$ and regulates sodium transport in PTCs remain to be investigated. Extracellular ANG II has been shown to increase $[Ca^{2+}]_i$ by promoting extracellular Ca^{2+} influx or by $[Ca^{2+}]_i$ release from intracellular stores in renal epithelial cells (4,34); intracellular ANG II may mobilize $[Ca^{2+}]_i$ primarily via an intracellular mechanism(s).

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GRANTS

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Fig. 1. Intracellular microinjection of unlabeled or FITC-ANG II in single proximal tubule cells (PTC). *A*: phase contrast micrograph taken before insertion of a glass micropipette. *B*: micrograph taken after insertion of a glass micropipette. *C*: fluorescence micrograph before microinjection of FITC-ANG II. *D*: fluorescence micrograph 30 s after microinjection of FITC-ANG II. Note that FITC-ANG II was distributed throughout the cytoplasm. N, nucleus. Magnification: $\times 40$.

Fig. 2.

A: Western blot of angiotensin type 1 (AT₁) receptor (R) protein expression in PTCs. The 42-kDa protein, corresponding to the reported molecular mass of AT₁ receptor protein in the rat kidney (*lanes 1 and 2*), was inhibited by an AT₁ receptor-selective blocking peptide [AT₁R antibody (Ab) + blocking peptide (BP); *lane 3*]. Western blot of β-actin on the same membrane after stripping confirmed equal protein loading. B: semiquantitated data of Western blot. ***P* < 0.01 vs. AT₁R Ab only by unpaired *t*-test.

Fig. 3. Intracellular ANG II levels in PTCs and the effect of AT₁ receptor-mediated endocytosis of extracellular ANG II. Exposure of PTCs to extracellular ANG II (1 nM) increased intracellular ANG II, whereas losartan (Los, 10 μ M) blocked this effect. $P < 0.01$ vs. control (**) and vs. ANG II (++).

Fig. 4.

Effect of extracellular ANG II and cell-surface AT_1 receptors on peak intracellular calcium concentration ($[Ca^{2+}]_i$) signaling in PTCs. Extracellular losartan completely blocked the cell-surface AT_1 receptor-mediated $[Ca^{2+}]_i$ response to extracellular ANG II. *A*: control. *B*: ANG II (1 nM). *C*: ANG II + losartan (10 μ M). *D*: quantitative $[Ca^{2+}]_i$ levels. Red represents the highest level of calcium signaling (relative fluorescence levels), whereas black is the background. $P < 0.01$ vs. control (*) and vs. ANG II (#).

Fig. 5.

A–E: effect of intracellular microinjection of ANG II (1 nM, ~70–100 fl) on $[Ca^{2+}]_i$ responses in single PTC at baseline (0 s) and 15, 30, 60, and 120 s after microinjection of ANG II in the cells. *F*: relative levels of $[Ca^{2+}]_i$ signaling before and after microinjection of ANG II. Red represents the highest level of $[Ca^{2+}]_i$ responses, whereas black is the background. ** $P < 0.01$ vs. basal.

Fig. 6. Effect of intracellularly applied AT_1 receptor antagonist losartan (10 μ M) or AT_2 receptor antagonist PD-123319 (PD, 10 μ M) on peak $[Ca^{2+}]_i$ responses to ANG II microinjection in single PTC. Losartan abolished ANG II-induced $[Ca^{2+}]_i$ responses, whereas PD-123319 had no effect. ** $P < 0.01$ vs. control without ANG II microinjection. ++ $P < 0.01$ vs. ANG II microinjection.

Fig. 7.

A–F: time-dependent $[Ca^{2+}]_i$ responses to microinjected ANG II (1 nM, 70–100 fl) in the absence of extracellular Ca^{2+} in a representative PTC. G: removal of extracellular Ca^{2+} from the perfusate had no significant effect on peak $[Ca^{2+}]_i$ responses to intracellular ANG II. Red represents the highest level of $[Ca^{2+}]_i$ responses, whereas black is the background. ** $P < 0.01$ vs. control.

Fig. 8. Peak $[Ca^{2+}]_i$ responses to microinjected ANG II (1 nM, 70–100 fl) in single PTCs pretreated with thapsigargin (1 μ M; **A**) to deplete intracellular $[Ca^{2+}]_i$ stores or with the phospholipase C inhibitor U-73122 (1 μ M; **B**). Thapsigargin and U-73122 abolished $[Ca^{2+}]_i$ responses to microinjected ANG II. $P < 0.01$ vs. control (**) and vs. ANG II (++)