Angiotensin II Stimulates Thick Ascending Limb Superoxide Production via Protein Kinase Cα-dependent NADPH Oxidase Activation*

Received for publication, January 29, 2010, and in revised form, April 22, 2010. Published, JBC Papers in Press, May 6, 2010, DOI 10.1074/jbc.M110.109157

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Angiotensin II (Ang II) stimulates thick ascending limb (TAL) O$_2^-$ production, but the receptor(s) and signaling mechanism(s) involved are unknown. The effect of Ang II on O$_2^-$ is generally attributed to the AT$_1$ receptor. In some cells, Ang II stimulates protein kinase C (PKC), whose α isoform (PKCα) can activate NADPH oxidase. We hypothesized that in TALs, Ang II stimulates O$_2^-$ via AT$_1$ and PKCα-dependent NADPH oxidase activation. In rat TALs, 1 nM Ang II stimulated $O_2^-$ from 0.76 ± 0.17 to 1.97 ± 0.21 nmol/min/mg (p < 0.001). An AT$_1$ antagonist blocked the stimulatory effect of Ang II on O$_2^-$ (0.87 ± 0.25 nmol/min/mg; p < 0.006), whereas an AT$_2$ antagonist had no effect (2.16 ± 0.133 nmol/min/mg; p < 0.05 versus vehicle). Apocynin, an NADPH oxidase inhibitor, blocked Ang II-stimulated O$_2^-$ by 90% (p < 0.01). Ang II failed to stimulate O$_2^-$ in TALs from p47phox$^{-/-}$ mice (p < 0.02). Monitored by fluorescence resonance energy transfer, Ang II increased PKC activity from 0.02 ± 0.03 to 0.13 ± 0.02 arbitrary units (p < 0.03). A general PKC inhibitor, GF109203X, blocked the effect of Ang II on O$_2^-$ (1.47 ± 0.21 versus 2.72 ± 0.47 nmol/min/mg with Ang II alone; p < 0.03). A PKCα- and β-selective inhibitor, Go6976, also blocked the stimulatory effect of Ang II on O$_2^-$ (0.59 ± 0.15 versus 2.05 ± 0.28 nmol/min/mg with Ang II alone; p < 0.001). To distinguish between PKCα and PKCβ, we used tubules expressing dominant-negative PKCα or -β. In control TALs, Ang II stimulated O$_2^-$ by 2.17 ± 0.44 nmol/min/mg (p < 0.001). In tubules expressing dominant-negative PKCα, Ang II failed to stimulate O$_2^-$ (change: -0.30 ± 0.27 nmol/min/mg). In tubules expressing dominant-negative PKCβ1, Ang II stimulated O$_2^-$ by 2.08 ± 0.69 nmol/min/mg (p < 0.002). We conclude that Ang II stimulates TAL O$_2^-$ production via activation of AT$_1$ receptors and PKCα-dependent NADPH oxidase.

The reactive oxygen species superoxide (O$_2^-$) plays an important role in the regulation of kidney function (1–3). O$_2^-$ decreases renal blood flow by constricting renal vessels (4), reduces glomerular filtration rate by enhancing tubuloglomerular feedback (5) and also promotes salt reabsorption along the nephron (6, 7). Excessive O$_2^-$ generation within the kidneys contributes to the development of hypertension (8), renal damage (9, 10), and atherosclerosis (11, 12). Thus clarifying the mechanisms that regulate O$_2^-$ production within the kidney may help us understand the etiology and pathophysiology of many diseases and develop new targets for treatment.

O$_2^-$ can be generated by several types of cells within the kidney (13, 14); however, it is primarily produced by the medullary thick ascending limb of the loop of Henle (TAL) (14). In the TAL, O$_2^-$ production can be stimulated by several factors, including Ang II (15, 16). Ang II can activate two types of receptors: AT$_1$ and AT$_2$. Activation of AT$_1$ is associated with the salt-retaining and pro-hypertensive actions of Ang II (17, 18). In the TAL, Ang II acutely stimulates O$_2^-$ production (15), but neither the receptor nor the signaling cascade involved has been identified.

O$_2^-$ can be produced by NADPH oxidase, xanthine oxidase, and the mitochondria (19). In the absence of Ang II stimulation, NADPH oxidase appears to be the main source in the renal medulla (20), particularly the TAL (14, 21, 22); however, the source of O$_2^-$ in the TAL during Ang II stimulation is still unknown.

In many types of cells, including TAL cells, activation of protein kinase C (PKC) has been shown to stimulate O$_2^-$ production in response to different stimuli, including Ang II (23, 24, 25). The PKC family of serine/threonine kinases is composed of many isoforms, some of which are expressed in the TAL, including PKCα, -β, -δ, -ε, and -ζ (26, 27). Yang et al. have shown that in the TAL PKCα mediates the enhanced O$_2^-$ levels observed during diabetes (24). However, to our knowledge there have been no studies investigating whether PKC mediates the stimulatory effect of Ang II on TAL O$_2^-$ production or the isoform(s) involved. We hypothesized that Ang II binds to the AT$_1$ receptors, activating PKCα, which in turn stimulates NADPH oxidase activity, enhancing O$_2^-$ production by the TAL.

EXPERIMENTAL PROCEDURES

Animals—Male Sprague-Dawley rats (Charles River, Kalamazoo, MI) were fed a diet containing 0.22% sodium and 1.1% potassium (Purina, Richmond, IN) for at least 7 days. Wild-type and p47phox$^{-/-}$ knock-out mice (Jackson Laboratories, Bar Harbor, ME), were fed regular chow for at least 7 days. On

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*This work was supported, in whole or in part, by National Institutes of Health Grants HL-70985 and HL-028982 (to J. L. G.).

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2 The abbreviations used are: TAL, thick ascending limb of the loop of Henle; Ang II, angiotensin II; PKC, protein kinase C; FRET, fluorescence resonance energy transfer; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; CMV, cytomegalovirus; HA, hemagglutinin; dn, dominant negative.
the day of the experiment, animals were anesthetized with ketamine (100 mg/kg body weight, intraperitoneally) and xylazine (20 mg/kg body weight, intraperitoneally). All protocols were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee.

**Medullary TAL Suspensions**—TAL suspensions were obtained from rats weighing 150–220 g as described previously (28). This procedure yields a suspension of TALs that is >90% pure (29), so that contamination by other types of cells in our preparation was minimal or absent.

**Measurement of $O_2^-$ Production**—200-μl aliquots of rat TAL suspensions were placed in glass tubes, and HEPS-buffered physiological saline (130 mM NaCl, 2.5 mM NaH$_2$PO$_4$, 4 mM KCl, 1.2 mM MgSO$_4$, 6 mM alanine, 1 mM Na$_3$ citrate, 5.5 mM glucose, 2 mM Ca$^{2+}$ (lactate)$_2$, and 10 mM HEPS (pH 7.4)) was added for a final volume of 1 ml. The whole suspension was used when TALs were obtained from mice. N,N’-Dimethyl-9,9'-bicacidinium dinitrate (Lucigenin, Sigma-Aldrich) was added to the suspensions to give a final concentration of 5 μM. When investigating the effects of apocynin (4-hydroxy-3-methoxyacetophenone, 10 μM, Sigma-Aldrich), GF109203X (100 nM, Enzo Life Sciences, Plymouth Meeting, PA), or Gö6976 (100 nM, Enzo Life Sciences), these were added to the tube, and the volume of physiological saline was adjusted accordingly. When using the Ang II receptor antagonists PD123319 (1 μM, Parke-Davis, Ann Arbor, MI) and losartan (1 μM, Merck, Rahway, NJ), these were added to the tubules 5 min before Ang II (1 nM, Bachem, Torrance, CA). Tubules were incubated for 10 min at 37 °C and then placed in a luminometer (model FB1/110,100/H, 11001/H, /H11022/H, 1200/H11003/H). After an overnight transfer, the polyvinylidine difluoride membrane was blocked in a buffer containing 20 mM Tris, 137 mM NaCl, 5% nonfat dried milk, and 0.1% Tween 20 (TBS-T) and incubated for 1 h at room temperature and then incubated with either a 1:1,000 dilution of mouse monoclonal anti-HA antibody (Abgent, San Diego, CA), 1:1,000 dilution of the CMV promoter, and then the CMV promoter, and sent to ViraQuest (North Liberty, IA) for viral production.

**Dominant Negative PKC Isoforms**—The HA (hemagglutinin)-tagged dominant negative PKCα and PKCβ1 plasmids (dn-PKCα and dn-PKCβ1) were kindly provided by Dr. Jae-Won Soh, Biomedical Research Center for Signal Transduction Networks, Incheon, Korea. The dominant negatives consist of kinase-dead PKC isoforms, generated by single amino acid substitution within the kinase domain. Sequences encoding for both proteins were subcloned into a shuttle vector containing the CMV promoter. Plasmids were sent to ViraQuest for adenoviral production.

**In Vivo Gene Delivery of CKAR and dn-PKCI Isoforms**—TALs were transduced in vivo with recombinant replication-deficient adenoviruses expressing the dn-PKCa, dn-PKCI, or CKAR sequence as we reported previously (31, 32). Briefly, kidneys of a 95- to 105-g rat were exposed via a flank incision, and the renal artery and vein were clamped. Four 20-μl virus injections (1 × 10$^{12}$ particles/ml) were made along the longitudinal axis at a flow rate of 20 μl/min. The renal vessels were unclamped; kidneys were returned to the abdominal cavity, the muscle incision was sutured, and the skin was clipped. Because we previously found that maximum expression occurred 3–5 days after injection of the adenovirus (32, 33), all experiments were performed within these time points. Expression of the dominant negatives was confirmed by Western blots.

**Expression of dn-PKCa and -β**—Western blots were performed as routinely done in our laboratory (28, 29). Briefly, 40 μg of TAL suspension homogenates was loaded onto an 8% polyacrylamide gel, and electrophoresis was performed for 2 h at 92 mV. After an overnight transfer, the polyvinylidine difluoride membrane was blocked in a buffer containing 20 mM Tris, 137 mM NaCl, 5% nonfat dried milk, and 0.1% Tween 20 (TBS-T) and 5% milk for 1 h at room temperature and then incubated with either a 1:1,000 dilution of mouse monoclonal anti-HA antibody (Abgent, San Diego, CA), 1:1,000 dilution of a mouse anti-PKCa antibody (BD Biosciences, San Jose, CA) or a 1:250 dilution of a mouse anti-PKCI antibody (BD Biosciences) for 1 h at room temperature. The membrane was washed using TBS-T and incubated for another hour with a 1:1,000 dilution of the appropriate IgG conjugated to horseradish peroxidase (Amersham Biosciences) for 1 h at room temperature. The reaction products were detected using a chemiluminescence kit (Amersham Biosciences) and by exposure to Fuji RX film.

**Measurements of PKC Activity by FRET**—On the day of the experiment, TAL suspensions were obtained from CKAR-transduced kidneys as indicated above and 1/5 of the suspension was seeded in a temperature-controlled chamber and warmed to 37 °C. The flow rate of the bath was 0.3 ml/min. During the 30-min equilibration period, images were acquired (100 × oil objective, numerical aperture: 1.3) by alternately exciting CFP (442 nm) and YFP (514 nm) and monitoring YFP emission at 540 nm to determine expression of the FRET sensor and highlight regions of interest. During the control period, CFP/YFP emission ratios were measured by exciting CFP at 442 nm once a minute for 5 min and simultaneously monitoring CFP and YFP emissions at 440–480 (CFP) and 540–545 nm (YFP). At the end of the control period, Ang II was added to the bath and CFP/YFP monitored once every minute for 15 min. The averages corresponding to the 5-min control period and the last 5 min of the experimental period were compared.
confirm that the YFP signal was due to FRET, control experiments were performed by photobleaching CFP and measuring the decrease in YFP emission. Images were acquired using the same settings (laser intensity, detector gain and offset, resolution, and exposure time).

Statistics—All statistical analyses were performed by the Biostatistics Department of Henry Ford Hospital. Results are expressed as mean ± S.E. Data were analyzed using Student’s t-tests. A version designed for unequal standard deviations was used when necessary. Some comparisons were studied using contrast statements. When multiple testing was involved, Hochberg’s method was used.

RESULTS

We first investigated the effect of Ang II on TAL O$_2$ production. When rat TAL suspensions were incubated for 10 min in vehicle (0.005% acetic acid in water), O$_2$ production was $0.76 \pm 0.17$ nmol/min/mg. However, in TALs treated with Ang II (1 nM for 10 min) it increased to $1.97 \pm 0.21$ nmol/min/mg ($p < 0.001$; $n = 11$), 159% stimulation (Fig. 1). These data suggested that Ang II stimulates O$_2$ production by TALs.

To investigate which angiotensin receptor mediates the effect of Ang II on O$_2$ production, we used pharmacological inhibitors of AT$_1$ and AT$_2$. In the presence of losartan, an AT$_1$ receptor antagonist (1 μM), Ang II (1 nM) failed to stimulate O$_2$ production ($0.87 \pm 0.25$ nmol/min/mg; $p < 0.006$ versus Ang II alone; $n = 6$) by rat TALs. However, when we used PD 123319, an AT$_2$ receptor antagonist (1 μM), Ang II raised O$_2$ production to $2.16 \pm 0.13$ nmol/min/mg ($p < 0.05$ versus vehicle; $n = 5$) (Fig. 1). In different sets of experiments, neither losartan nor PD 123319 changed basal O$_2$ levels ($0.78 \pm 0.38$ nmol/min/mg for baseline versus $0.80 \pm 0.06$ nmol/min/mg for losartan alone; $n = 3$ and $1.04 \pm 0.09$ nmol/min/mg for baseline versus $1.05 \pm 0.12$ nmol/min/mg for PD 123319 alone; $n = 3$). These data indicated that Ang II binds the AT$_1$ receptor to stimulate O$_2$ production by TALs.

Next we tested whether NADPH oxidase is the source of Ang II-stimulated O$_2$ in TALs using the NADPH oxidase inhibitor apocynin and TALs isolated from p47phox knock-out (−/−) mice. When rat TAL suspensions were incubated with vehicle (0.005% acetic acid), O$_2$ production was $1.54 \pm 0.31$ nmol/min/mg. With Ang II (1 nM for 10 min) it increased to $4.10 \pm 0.69$ nmol/min/mg ($p < 0.001$ versus vehicle; $n = 4$). However, when we added apocynin (10 μM), Ang II failed to stimulate O$_2$ production (1.49 ± 0.32 nmol/min/mg; $p < 0.01$ versus Ang II alone; $n = 5$) (Fig. 2A). In a different set of experiments, apocynin alone significantly reduced basal O$_2$ production by 80% ($p < 0.005$; $n = 5$). To make sure the effect of apocynin was due to specific inhibition of NADPH oxidase, we performed experiments using tubules isolated from p47phox−/− mice. In the presence of Ang II, O$_2$ production was $1.45 \pm 0.12$ nmol/min/mg in wild-type controls but undetectable in TALs from p47phox−/− mice ($0.00 \pm 0.32$ nmol/min/mg) ($p < 0.02$; $n = 4$ for each group) (Fig. 2B). These data indicate that NADPH oxidase is the primary source of O$_2$ in the TAL under both basal and Ang II-stimulated conditions.

To test whether Ang II directly enhances PKC activity in the rat TAL, we measured the effect of Ang II on PKC activity using FRET. In tubules expressing CKAR and incubated with vehicle (0.005% acetic acid) the CFP/YFP ratio was 0.02 ± 0.03 arbitrary unit. Upon adding Ang II (1 nM) to the same tubules,
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![Graph 3: Acute effect of 1 nm Ang II on total PKC activity by FRET. Rat thick ascending limbs expressing a PKC activity reporter were used.](image3)

![Graph 4: Effect of 100 nm GF109203X (a general PKC inhibitor) on the stimulatory effect of 1 nm Ang II for 10 min on $O_2^-$ production by rat thick ascending limbs.](image4)

![Graph 5: Effect of 100 nm Gö6976 (a selective PKCα and β1 inhibitor) on the stimulatory effect of 1 nm Ang II for 10 min on $O_2^-$ production by rat thick ascending limbs.](image5)

CFP/YFP increased to 0.13 ± 0.02 arbitrary unit ($p < 0.03$; $n = 6$) (Fig. 3). These data suggested that Ang II activates PKC activity in the TAL.

To determine whether activation of PKC is required for the stimulatory effect of Ang II on $O_2^-$ production, we used a general PKC inhibitor, GF109203X. In rat TALs 1 nm Ang II stimulated $O_2^-$ production to 2.72 ± 0.47 nmol/min/mg. However, with 100 nm GF109203X the effect of Ang II was significantly reduced (1.47 ± 0.21 nmol/min/mg; $p < 0.03$ versus Ang II alone; $n = 6$) (Fig. 4). In a different set of experiments, GF109203X did not change basal $O_2^-$ production (1.13 ± 0.15 nmol/min/mg for baseline versus 0.83 ± 0.12 nmol/min/mg for GF109203X alone; $n = 3$). These data indicate that Ang II stimulates TAL $O_2^-$ production by activating PKC.

To find out which PKC isoform(s) mediates Ang II-stimulated $O_2^-$ production, we used Gö6976, a PKCα- and β1-selective inhibitor. In rat TAL suspensions, 1 nm Ang II raised $O_2^-$ production to 2.05 ± 0.28 nmol/min/mg. However, when we added Gö6976 (100 nm) to the preparation, Ang II failed to stimulate $O_2^-$ production (0.59 ± 0.15 nmol/min/mg; $p < 0.001$; $n = 6$) (Fig. 5). In a different set of experiments, Gö6976 did not change basal $O_2^-$ production (1.17 ± 0.10 nmol/min/mg for baseline versus 1.10 ± 0.07 nmol/min/mg for Gö6976 alone; $n = 3$). These results suggested that Ang II stimulates TAL $O_2^-$ production by activating PKCα and/or PKCβ1.

To clarify the PKC isoform(s) involved, we transduced rat TALs in vivo so that they expressed either control DNA, dominant-negative PKCα (dn-PKCα) or dominant-negative PKCβ1 (dn-PKCβ1). Expression of the dominant negatives was maximal 3–5 days after adenoviral injection as assessed by Western blots. The dominant negatives are HA-tagged, kinase-dead mutants generated by a single point mutation within the kinase domain. Thus, their expression can be monitored by the presence of HA and also by an increase in total PKCα or -β (because the antibodies used for Western blot also recognize the mutants). We found >500% increase of HA expression compared with the non-injected kidney ($n = 4$ for dn-PKCα and $n = 3$ for dn-PKCβ1). In addition, total PKCα increased by 250% in dn-PKCα-injected versus non-injected kidney ($p < 0.004$; $n = 4$) and PKCβ by 293% dn-PKCβ1-injected versus non-injected kidney ($p < 0.08$; $n = 3$). All experiments were performed 3–5 days after adenoviral transduction. In control rat TALs incubated with vehicle (0.005% acetic acid), $O_2^-$ production was 1.42 ± 0.12 nmol/min/mg, and with 1 nm Ang II it rose to 3.58 ± 0.51 nmol/min/mg of protein ($p < 0.011$, $n = 5$) (Fig. 6). In contrast, in tubules expressing dn-PKCα, Ang II failed to stimulate $O_2^-$ (1.53 ± 0.67 nmol/min/mg; n.s. versus vehicle), whereas in tubules expressing dn-PKCβ1 Ang II raised $O_2^-$ production to 3.89 ± 0.37 nmol/min/mg (p < 0.002 versus vehicle; $n = 6$) (Fig. 6). Neither dn-PKC isoform had any effect on basal $O_2^-$ (vehicle-treated suspensions; black bars in Fig. 6). These data indicated that PKCα mediates the stimulatory effect of Ang II on $O_2^-$ production by TALs.

DISCUSSION

We hypothesized that Ang II acts on the AT1 receptor to stimulate $O_2^-$ production by the TAL, and that this process involves stimulation of PKCα, which in turn activates NADPH oxidase. We found that: 1) Ang II stimulated rat TAL $O_2^-$ production, and this process was halted by blocking AT1 but not AT2; 2) the NADPH oxidase inhibitor apocynin blocked the
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Effect of vehicle or 1 nM Ang II for 10 min on O$_2^\bullet$ production by rat thick ascending limbs expressing control, dn-PKC$\alpha$, or dn-PKC$\beta_1$. n = 5–6.

stimulatory effect of Ang II on O$_2^\bullet$ production; 3) Ang II-induced O$_2^\bullet$ production was blunted in TALs isolated from p47$^{phox}^{-/-}$ mice; 4) in rat TALs Ang II increased PKC activity as measured by FRET; 5) in rat TALs, Ang II-induced O$_2^\bullet$ production was blocked by a general PKC inhibitor as well as by an inhibitor of both PKC$\alpha$ and $\beta_1$; and finally 6) Ang II-induced O$_2^\bullet$ production was reduced in rat TALs expressing dn-PKC$\alpha$ but intact in tubules expressing dn-PKC$\beta_1$.

We found that AT$_1$ mediated the stimulatory effect of Ang II on O$_2^\bullet$ production in the rat TAL but AT$_2$ did not, consistent with several studies conducted with other tissues. Fu et al. (33) recently reported that, in freshly isolated macula densa cells, Ang II stimulated O$_2^\bullet$ production, and this effect was blocked by the AT$_1$ antagonist losartan. Jaimes et al. (13) found that Ang II stimulated O$_2^\bullet$ production in cultured mesangial cells, and this effect was blocked by an AT$_1$ antagonist. Plumb et al. (35) reported that Ang II stimulated O$_2^\bullet$ production in human platelets, and this effect was blunted by an AT$_1$ receptor antagonist. Although we recently reported that Ang II acts on AT$_2$ receptors to activate other signaling events in the TAL (34), in the present study the AT$_2$ antagonist PD123319 had no effect on Ang II-stimulated O$_2^\bullet$ production, suggesting that AT$_2$ receptors do not play a role in AT$_1$-stimulated O$_2^\bullet$ production in the TAL. Thus it seems likely that activation of each receptor subtype leads to stimulation of independent signaling pathways.

We also questioned whether NADPH oxidase is the source of O$_2^\bullet$ in Ang II-stimulated TALs. NADPH oxidase is an enzymatic complex that comprises five components: p40$^{phox}$, p47$^{phox}$, p67$^{phox}$, p22$^{phox}$, and NOX (35). Under basal conditions p40$^{phox}$, p47$^{phox}$ and p67$^{phox}$ are located in the cytosol as a complex. Upon stimulation, p47$^{phox}$ becomes phosphorylated and the cytosolic complex translocates to the cell membrane, where it assembles with p22$^{phox}$ and NOX and generates O$_2^\bullet$ (36). Thus p47$^{phox}$ is essential for activation of NADPH oxidase. We found that apocynin, which inhibits translocation of p47$^{phox}$ to the plasma membrane, completely inhibited Ang II-stimulated O$_2^\bullet$ production, suggesting that in the TAL all Ang II-stimulated O$_2^\bullet$ is generated by NADPH oxidase. In addition, we found that apocynin alone significantly reduced basal levels of O$_2^\bullet$ in the rat TAL, suggesting that NADPH oxidase generates basal O$_2^\bullet$ levels. We recognize that the basal level of O$_2^\bullet$ on Fig. 2 is higher than what we found for Fig. 1. The explanation for such discrepancy is unknown; however, it should be mentioned that experiments were done during different times of the year, and this may influence production of O$_2^\bullet$ by the rat TAL.

To make sure the effect of apocynin was specifically due to inhibition of NADPH oxidase, we tested TALs isolated from p47$^{phox}^{-/-}$ mice and found that they had low basal levels of O$_2^\bullet$, which were not stimulated by Ang II, indicating that: 1) p47$^{phox}$ is required for Ang II-stimulated O$_2^\bullet$ production in TALs and 2) it maintains basal TAL levels of O$_2^\bullet$. We were unable to uncover any compensatory mechanism that enables O$_2^\bullet$ to be generated under both basal and stimulated conditions in these mice. We recognize that the results obtained in mice cannot necessarily be extrapolated to rats. In fact, the degree of Ang II-stimulated O$_2^\bullet$ production was lower in mice compared with rats. However, the p47$^{phox}^{-/-}$ mice were used as a tool to investigate the involvement of NADPH oxidase so that we did not rely only on pharmacological inhibition. These findings are consistent with data from Li et al. (14) showing that in unstimulated TALs NADPH oxidase is the major source of O$_2^\bullet$ production. In addition, a recent report from our laboratory demonstrated that luminal flow stimulated TAL O$_2^\bullet$ production via activation of NADPH oxidase (37). Thus both mechanical and humoral factors are capable of activating NADPH oxidase and thereby enhancing O$_2^\bullet$ production by the TAL. Taken together, these data confirm that NADPH oxidase is the main source of O$_2^\bullet$ in the TAL under both basal and stimulated conditions.

To test the involvement of PKC in Ang II-stimulated O$_2^\bullet$ production, we first measured total PKC activity in real-time by FRET. We found that Ang II acutely increased PKC activity within 3 min after adding Ang II. When we applied the general PKC pharmacological inhibitor GF109203X, we found that it inhibited Ang II-generated O$_2^\bullet$ production, indicating that PKC activity is necessary for Ang II to stimulate O$_2^\bullet$ production by TALs. These data are consistent with recent reports from other investigators suggesting that PKC contributes to enhanced O$_2^\bullet$ production in the kidney. Zhang et al. (38) showed that Ang II constricted pericytes within the vasa recta via a mechanism requiring activation of PKC. Yang et al. reported that PKC activation is responsible for the increased O$_2^\bullet$ in diabetic rat TALs (24). More recently, we have shown that luminal flow stimulates O$_2^\bullet$ production via activation of PKC in isolated perfused TALs (37). Thus activation of PKC appears to be an important mechanism leading to enhanced O$_2^\bullet$ within the kidney.

The PKC protein family is composed of at least eight members, of which five have been shown to be expressed in the TAL: PKC$\alpha$, $\beta$, $\delta$, $\epsilon$, and $\xi$ (26, 27). To find out which isoform(s) might be involved in Ang II-induced O$_2^\bullet$ production, we used G906976, which inhibits both PKC$\alpha$ and $\beta$. We found that G906976 completely blocked Ang II-induced O$_2^\bullet$ production. Because we know of no pharmacological inhibitor specific enough to target only PKC$\alpha$ or $\beta$, we used adenoviral-mediated transduction of dn-PKC$\alpha$ or $\beta$. We found that Ang II stimulated O$_2^\bullet$ production both in controls and in dn-PKC$\beta_1$-
transduced TALs; however, it had no effect on TALs transduced with dn-PKCa. Taken together, these data indicate that Ang II stimulates NADPH oxidase-derived O$_2^-$ production in the TAL by activating PKCa.

We conclude that in the TAL, Ang II acts on the AT$_1$ receptor, activating PKCa, which in turn stimulates first NADPH oxidase and ultimately O$_2^-$ production, although the exact signaling pathway remains unknown. The AT$_1$ receptors are coupled to G$_q$ and G$_i$ proteins. Activation of G$_q$ enhances diacylglycerol production and stimulates intracellular Ca$^{2+}$ (39, 40), either of which is capable of activating the classic PKC isoforms $\alpha$ and $\beta$ (41). In other cells, O$_2^-$ stimulation by AT$_1$ activation also participates in both Ang II-induced NADPH oxidase activation and O$_2^-$ production (42). Both of these pathways could mediate AT$_1$-dependent activation of PKCa in the TAL. In addition, Ang II has been reported to activate the small GTPase Rac (43), whose trafficking and translocation to the plasma membrane play an important role in activation of NADPH oxidase (44). In the TAL, Rac mediates NaCl-induced O$_2^-$ production (45). Thus Rac could also participate in both Ang II-induced NADPH oxidase activation and O$_2^-$ production in the TAL.

In summary, in TALs Ang II acts on the AT$_1$ receptor to stimulate O$_2^-$ production by activating PKC and Rac, whose trafficking and translocation to the plasma membrane play an important role in activation of NADPH oxidase (42). According to our data, PKC and Rac are sensitive to increases in intracellular Ca$^{2+}$ (26). According to our data, PKC and Rac are sensitive to increases in intracellular Ca$^{2+}$ (26).

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