

Angiotensin II Regulation of the Na⁺ Pump Involves the Phosphatidylinositol-3 Kinase and p42/44 Mitogen-Activated Protein Kinase Signaling Pathways in Vascular Smooth Muscle Cells

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This investigation used primary cultured rat vascular smooth muscle cells to examine angiotensin II (Ang II) regulation of Na⁺, K⁺-ATPase (Na⁺ pump) activity, and Na⁺ pump α_1 - and β_1 -subunit gene transcription. This regulation was mediated through both phosphatidylinositol-3 kinase (PI3K) and p42/44 mitogen-activated protein kinase (p42/44^{MAPK}) signaling pathways. Both acute (10 min) and prolonged (24 h) treatment with Ang II stimulated Na⁺ pump activity. Also, prolonged exposure to Ang II (24 h) increased promoter transcription of the Na⁺ pump α_1 - and β_1 -subunits. Furthermore, PI3K activities because well because p42/44^{MAPK} phosphorylation were increased within 10 min after Ang II treatment. To determine whether these stimulatory activities of Ang II are acting through Ang II receptors 1 and/or 2 (AT₁, AT₂), cells were pretreated with either AT₁ receptor blocker losartan or the AT₂ receptor blocker PD 123,319. Indeed, these treatments prevented the stimulatory effect of Ang II on Na⁺ pump activity at both acute and 24-h time points. Furthermore, the Ang II-stimulated α_1 -subunit promoter transcription was inhibited by losartan but not by the AT₂ receptor blocker. These results indicate that Ang II acts through both the AT₁ and AT₂ receptor to up-regulate Na⁺ pump activity; however, Ang II regulates α_1 -gene transcription through AT₁ but not AT₂ receptors. It was also observed that the Ang II-stimulated β_1 -subunit gene transcription is not mediated through either AT₁ or AT₂ receptors. To examine whether the Na⁺/H⁺ exchanger is involved in Ang II-stimulated Na⁺ pump activity, cells were pretreated with amiloride, a specific inhibitor of the Na⁺/H⁺

exchanger. This pretreatment prevented 24 h, but not acute, Ang II-stimulated Na⁺ pump activity. The 24-h Ang II-stimulated α_1 -subunit promoter transcription was also inhibited by amiloride. This suggests that the prolonged effect of Ang II on Na⁺ pump activity is dependent on increased Na⁺/H⁺ exchange. Because Ang II treatment for 10 min increased PI3K activity because well because p42/44^{MAPK} phosphorylation, studies were performed to determine the involvement of PI3K and p42/44^{MAPK} signaling pathways in both Ang II-stimulated Na⁺ pump activity and α_1 - and β_1 -gene transcription. Cells were pretreated with either the PI3K inhibitor wortmannin or the p42/44^{MAPK} inhibitor PD 98059. Ang II-stimulated PI3K or p42/44^{MAPK} activity was inhibited by these pretreatments. Furthermore, pretreatment of cells with the PI3K inhibitors wortmannin and LY29404 or the MAPK inhibitors U0126 and PD 98059 were all observed to inhibit Ang II-stimulated Na⁺ pump activity. To more specifically determine the role of PI3K in Ang II-regulation of α_1 - and β_1 -gene transcription, cells were cotransfected with a dominant-negative p85 construct. Cotransfection with dominant-negative p85 reduced Ang II-stimulated α_1 -but not β_1 -gene transcription in vascular smooth muscle cells. These results indicate that Ang II acts through PI3K/p42/44^{MAPK} signaling pathways to up-regulate Na⁺ pump activity and α_1 -gene transcription and that Ang II-regulated β_1 -gene transcription is not mediated through either PI3K or p42/44^{MAPK} signaling pathways. (*Endocrinology* 145: 1151–1160, 2004)

ANGIOTENSIN II (ANG II), the biologically active component of the renin-angiotensin system (RAS), acts through two Ang II receptor subtypes, the AT₁ and the AT₂ receptors, which mediate cardiovascular and other actions of Ang II. The two receptors are comprised of seven transmembrane glycoproteins with a 30% sequence homology (1, 2). The receptor binding sites for agonist and nonpeptide antagonist ligands have been defined, and AT₁ antagonists are

Abbreviations: Δ , Dominant-negative; Ang II, angiotensin II; AT₁, AT₂, Ang II receptors 1 and 2; EGF, epithelial growth factor; IRS, insulin receptor substrate; PI3K, phosphatidylinositol-3 kinase; RAS, renin-angiotensin system; VSMC, vascular smooth muscle cell.

Endocrinology is published monthly by The Endocrine Society (<http://www.endo-society.org>), the foremost professional society serving the endocrine community.

effective because antihypertensive agents that are well tolerated (1, 2). The development of specific nonpeptide receptor antagonists has led to major advances in the physiology and therapy of the RAS. All classic physiological effects of Ang II, such as vasoconstriction, cell proliferation, aldosterone and vasopressin release, sodium and water retention, and sympathetic facilitation, are mediated by the AT₁ receptor (1, 2). The AT₂ receptor, in contrast, is up-regulated in pathological conditions, and it counteracts several of the growth responses initiated by the AT₁ and growth factor receptors.

In the vascular smooth muscle cell (VSMC), Ang II regulates via VSMC AT₁ receptors, many processes including the state of contractility/relaxation (3–5). AT₁ receptors are predominantly coupled to G proteins and signal through phos-

pholipases, inositol phosphates, calcium channels, and a variety of serine/threonine and tyrosine kinases (1, 2, 4). In this regard, Ang II can activate phosphatidylinositol 3-kinase (PI3K), the AT₁ receptor. After Ang II-induced phosphorylation of PI3K's regulatory p85 subunit, p85 forms complexes with specific phosphotyrosines of either growth factors or adapter proteins such as insulin receptor substrate (IRS)-1, thereby influencing vascular tone and numerous VSMC functions (5, 6). This protein-protein interaction allows PI3K's catalytic p110 subunit to phosphorylate phosphoinositides at the 3' position of the inositol ring to generate 3-phosphoinositides. These lipids then serve as intermediates for specific downstream signal transduction events, leading to a multitude of biological responses (5–8). Another important serine/threonine protein kinase activated by Ang II is p42/44 MAPK (p42/44^{MAPK}) (9, 10). The p42/44^{MAPK} signaling pathway is a distinct serine-threonine kinase cascade consisting of three enzymes: MAPK kinase kinase, MAPK kinase (MAPKK, MEK, MKK), and MAPK. Upstream activators of the MAPK pathways include small GTPases of the Ras family, and downstream effectors include transcription factors and other kinases (11, 12).

The Na⁺, K⁺-ATPase (Na⁺ pump) is a plasma membrane enzyme that plays a crucial role in VSMC homeostasis, and it maintains Na⁺ and K⁺ gradients between the intra- and extracellular milieu that are important for the maintenance of cell volume and tone (13). Structurally, the minimal units of the Na⁺ pump are two major polypeptides, the α - and the β -subunits having different isoforms, 4 α (α_1 , α_4) and 3 β (β_1 , β_3) (13, 14). The subunits are responsible for the catalytic and transport properties of the enzyme because it contains binding sites for cations and ATP, and it also includes a phosphorylation site (14, 15). The β -subunits are involved in the docking of the Na⁺ pump to the plasma membrane (13, 14).

Despite the physiological and pathophysiological importance of Ang II, possible signal transduction pathways involved in its regulation of the Na⁺ pump remains poorly understood (15, 16). Thus, the aim of this investigation was to further elucidate the signaling mechanisms employed by Ang II in modulating the regulation of Na⁺ pump activity in VSMCs with particular emphasis on both PI3K and MAPK signaling pathways.

Materials and Methods

VSMC preparation and culture

VSMCs were isolated from Sprague Dawley rat thoracic aorta by enzymatic dissociation as described previously (17, 18). Primary cultured cells were grown (37 C in 95% humidified air and 5% CO₂) in glucose-free DMEM (without addition of insulin or growth factors) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Serial passages (through passage 7) of VSMCs were obtained by treating confluent cultures with 0.2% trypsin-EGTA in Ca²⁺- and Mg²⁺-free Hanks' balanced salt solution (Sigma Chemical, St. Louis, MO). Cells were characterized as VSMCs by a hill-and-valley pattern displayed at confluence and by positive immunostaining with a monoclonal antibody to VSMC-specific α -actin (Sigma). The Animal Research Committee of the State University of New York Health Science Center at Brooklyn approved the animal experimentation described within this report.

Ouabain-sensitive ⁸⁶Rb uptake

Na⁺ pump activity was determined by measuring ouabain (1 mM)-sensitive ⁸⁶Rb flux based on the principle that ⁸⁶Rb transport displays identical kinetics to K⁺ (19). VSMCs were seeded onto 24-well tissue culture plates at a density of 10,000 cells/well in DMEM/9% fetal bovine serum. Medium was changed every 2–3 d until the cells were 100% confluent (5–7 d). On the day of the experiment, cells were washed three times with RPMI 1640 [containing 102 mM NaCl, 5.6 mM Na₂HPO₄, 5.4 mM KCl, 0.4 mM CaCl₂/2H₂O, 0.4 mM MgSO₄/7H₂O, 10 mM glucose, and 24 HEPES (pH 7.4)] and acclimated in the final wash for 20 min. The treatment paradigm consisted of Ang II for 10 min or 24 h followed by the incubation in the presence or absence of 1 mM ouabain for 10 min in RPMI 1640. Then 1 μ Ci ⁸⁶Rb was added to all wells, and ⁸⁶Rb flux was allowed to proceed for 10 min. ⁸⁶Rb flux was terminated by addition of ice-cold 100 mM MgCl₂ followed by three washes with the same solution. Cells were solubilized with NaOH and neutralized with HCl, and ⁸⁶Rb uptake was quantified by liquid scintillation counting. Na⁺ pump activity was calculated as a percentage of ouabain-sensitive uptakes *vs.* total ⁸⁶Rb uptake and expressed as percent of control. All experiments were completed using three to six replicates per treatment per experiment.

Preparation of cell lysates

Quiescent VSMCs in 100-mm culture dishes were incubated with 100 nM Ang II, and Ang II-induced responses were terminated by addition of ice-cold PBS. This dose of Ang II was been previously shown to maximally stimulate Na⁺ pump activity in VSMCs (16). Cells were rinsed again with PBS and lysed by the addition of 1 ml lysis buffer [20 mM Tris HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM Na₄P₂O₇, 2 mM Na₃O₄, 1 mM β -glycerolphosphate, 1% Triton X-100] and the following protease inhibitors: 1 mM phenylmethylsulfonylfluoride, 10 μ g/ml leupeptin, and 5 μ g/ml aprotinin (Sigma). Cells were scraped and lysates were subjected to centrifugation at 13,000 rpm. Clarified supernatants were used fresh or stored at –70 C (20).

Immunoblot analysis

Equal amounts of protein (50 μ g) were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane in Tris-glycine transfer buffer containing 20% methanol in a trans-blot cell (Bio-Rad, Hercules, CA). Membranes were blocked in 5% instant nonfat dry milk in Tris-buffered saline [20 mM Tris, 137 mM NaCl (pH 7.6) containing 0.3% Tween 20], washed in Tris-buffered saline, and probed with primary antibody (dilute 1:1000) raised against the p42/44^{MAPK} kinase (Cell Signaling, Beverly, MA). The immunoblots were subsequently washed and incubated in (1: 5000) horseradish peroxidase-coupled antirabbit IgG antibody (Cell Signaling) for 1 h. The bound antibodies were visualized by enhanced chemiluminescence using the ECL system (Amersham, Piscataway, NJ) and exposure to X-OMAT film (Kodak, Rochester, NY). Signals were quantitated by a densitometry by using NIH 1.60 Software (National Institutes of Health, Bethesda, MD). Multiple exposure of each blot was performed to ensure that signals were within the linear range of the film.

Immunoprecipitation and assay for PI3K activity

Cell lysates prepared, as described above, were probed with an antiphosphotyrosine antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 C with gentle rocking. Protein A/G (20 μ l; 50% slurry, Santa Cruz) was added and samples were further incubated for an additional 2 h. The immune complexes were then recovered by centrifugation and used for measuring PI3K activity as previously described (17, 21). The reaction products were separated by thin-layer chromatography on oxalate-pretreated Silica Gel 60 plates in a solvent of chloroform/methanol/4 N ammonia (60:47:17). Cold propidium iodide, phosphatidylinositol phosphate, and phosphatidylinositol diphosphate were run as a standard and visualized by primulin staining. ³²P-labeled phosphatidylinositol 3-P products were measured using a STORM PhosphorImager and calculated by the IMAGEQUANT software (Molecular Dynamics, Sunnyvale, CA).

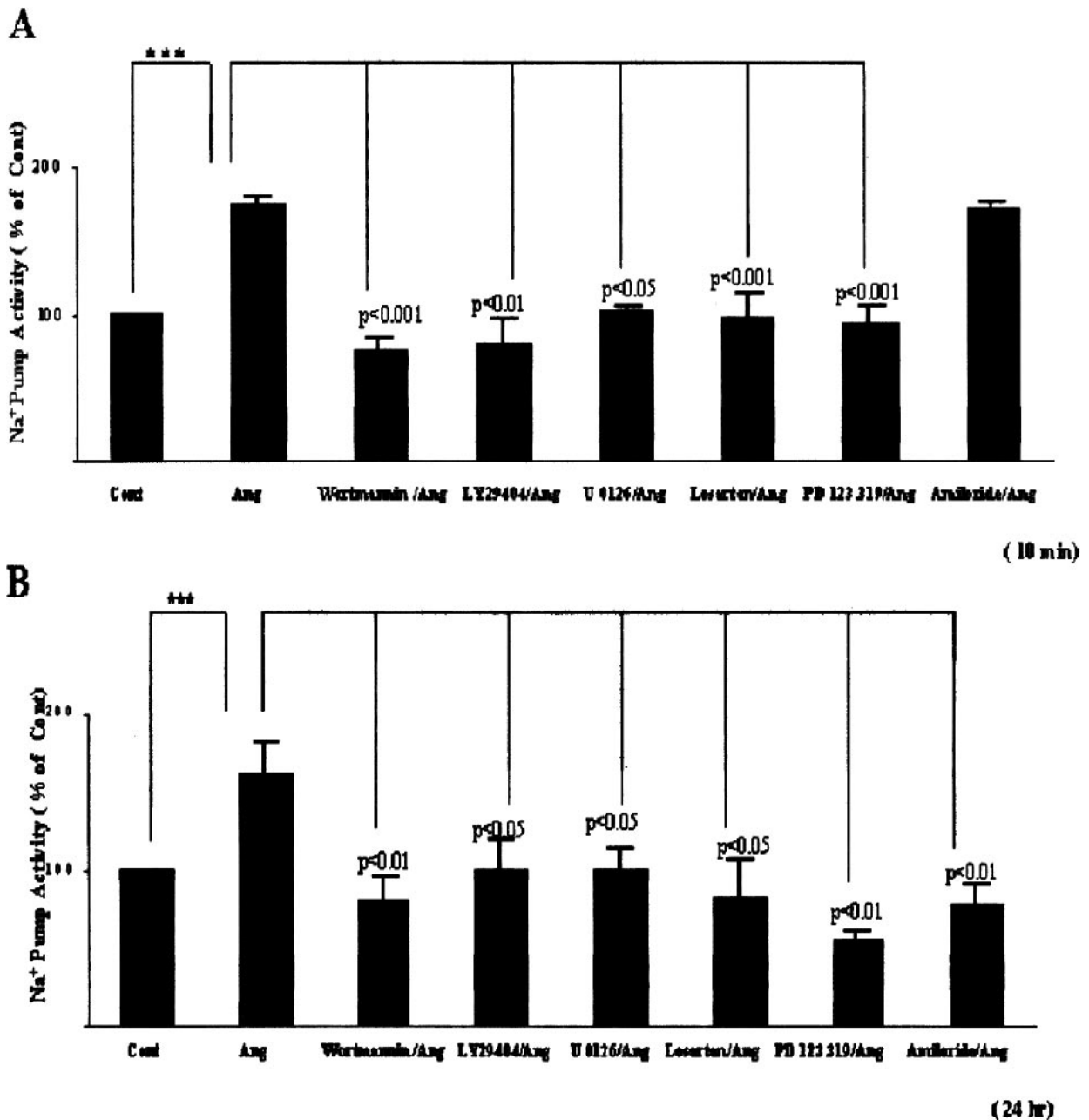


FIG. 1. Role of PI3K, p42/44^{MAPK}, AT₁, and AT₂ receptors, and Na⁺/H⁺ exchanger in Ang II-stimulated Na⁺ pump activity. Cells were treated with Ang II (100 nM; 10 min) (A) or 24-h (B) pretreatment with inhibitors: wortmannin (50 nM; 15 min), U0126 (10 μM; 15 min), PD 12339 (10 μM; 15 min), or losartan (1 μM; 15 min) before Ang II treatment. The results are expressed as percent of control (arbitrarily set at 100%). Results are mean SEM, n = 3–6 experiments performed in triplicate. ***, P < 0.001, indicates Ang vs. cont; the other P values shown are inhibitor-treated groups vs. Ang II-treated cells.

Transfection and luciferase assay

Cells (40–50% confluent) were transiently transfected using the cytofectene reagent (Bio-Rad) according to the manufacturer's protocol and a standardized method (17, 20, 22). Briefly, cells in 30-mm plates were transfected with 1 μg of a luciferase reporter plasmid contain a portion of Na⁺ pump α₁-subunit promoter (−1537 to +261) (17, 20) or 1 μg of a luciferase reporter plasmid contains a portion of the Na⁺ pump β₁-subunit promoter (−871 to +151) (17, 22). The cells were cotransfected with 20 ng luciferase-pRL-SV40 (Promega, Madison, WI) as an internal control. Four hours after transfection, the transfection mix was removed and cells were washed twice with serum-free media. In some experiments where indicated, VSMCs were pretreated for 15–30 min with the

stated concentrations of inhibitors (either wortmannin, PD 98059, losartan, or amiloride) before addition of fresh serum-free media in the absence or presence of Ang II (100 nM; 24 h). Cells were washed twice with ice-cold PB and lysed with passive lysis buffer (Promega). Lysates were analyzed for both firefly and Renilla luciferase activity using Promega dual-luciferase reporter assay kit. In some cases in which cells were not cotransfected with pRL-SV40 vector, only firefly luciferase was measured in aliquots containing equal amount of protein. Negative controls of mock-transfected cells and empty vector (pUSEamp, Promega) were incorporated in all experiments. To control for transfection efficiency, pGL3 control plasmid (Promega) containing the firefly luciferase was used in all experiments. In cotransfection experiments with

dominant-negative (Δ)p85 (lacking a binding site for the p110 catalytic subunit of PI3K), the cells were transfected with an additional 1 μ g of the plasmid and the total amount of DNA in transfected cells was kept constant by addition of the empty vector. Cleared cell-lysates were assayed for luciferase in an Optocomp1 single-sample luminometer. Activity of the Na⁺ pump-promoter reporter constructs was normalized to the activity of Renilla reporter.

Statistical analysis

Values are expressed as mean \pm SEM. Statistical significance was evaluated with nonparametric test (Mann-Whitney rank sum test) or ANOVA with the appropriate correction for multiple comparisons (Newman-Keuls method). $P < 0.05$ was considered significant. All comparisons are *vs.* control values, unless otherwise specified.

Results

Involvement of PI3K and p42/44^{MAPK} and role of Na⁺/H⁺ exchanger and Ang II-stimulated Na⁺ pump activity

It has been reported that Ang II (100 nM) stimulates Na⁺ pump activity in VSMCs with maximum effects within 10 min (17). Thus, this concentration of Ang II was used in our experiments to study both the acute (10 min) and prolonged (24 h) effects of Ang II on Na⁺ pump activity. Na⁺ pump activity was determined by measuring ouabain (1 mM)-sensitive ⁸⁶Rb influx (19). Both the acute and prolonged treatment significantly stimulated Na⁺ pump activity above control values by 72% (Fig. 1A) and 63% (Fig. 1B), respectively. To clarify the signaling pathway involved in Ang II regulation of Na⁺ pump activity, we examined the role of both PI3K and p42/44^{MAPK} signal transduction pathways. To determine whether PI3K is involved, VSMCs were first pretreated with the PI3K blockers wortmannin (50 nM; 30 min) or LY29404 (50 μ M; 15 min) before both acute and prolonged Ang II treatment. Ang II-stimulated Na⁺ pump activity was significantly decreased by wortmannin as well as LY 29404 treatments at both 10 min (Fig. 1A) and 24 h (Fig. 1B) without any significant effect on basal Na⁺ pump activity.

To investigate whether p42/p44^{MAPK} is involved in Ang II-stimulated Na⁺ pump activity, cells were pretreated with U 0126 (10 μ M; 15 min), a specific p42/44^{MAPK} inhibitor (23). U 0126 alone had no effect on basal Na⁺ pump activity (U0126 = 101.3%, control = 100%, $n = 3$) but did reduce both acute (Fig. 1A) and prolonged (Fig. 1B) Ang II-stimulated Na⁺ pump activity. These results indicate that activation of both PI3K and p42/44^{MAPK} pathways have a significant role in Ang II stimulation of Na⁺ pump activity in VSMCs.

The role of the Na⁺/H⁺ exchanger in Ang II stimulation of Na⁺ pump activity was also studied. In this regard, cells were pretreated with 5-(*N*-ethyl-*N*-isopropyl amiloride) (10 μ M; 10 min), a specific inhibitor of the Na⁺/H⁺ exchanger (15, 24). Inhibition of Na⁺/H⁺ exchange did not affect acute Ang II-stimulated Na⁺ pump activity (Fig. 1A) but did significantly diminish prolonged Ang II-stimulated Na⁺ pump activity (Fig. 1B), implicating a role for the Na⁺/H⁺ exchanger in Ang II's actions.

Role of AT₁ and AT₂ receptors in Ang II-stimulated Na⁺ pump activity

Because Ang II is known to mediate many of its biological effects in VSMCs through activation of the AT₁ receptor

(1–5), the effect of the specific AT₁ receptor antagonist losartan (25) on Na⁺ pump activity was examined. For these studies VSMCs were pretreated with losartan (1 μ M; 15 min) before both acute and prolonged Ang II treatment. The Ang II-induced Na⁺ pump activity was completely suppressed both acutely (Fig. 1A) and at 24 h (Fig. 1B) by pretreatment with this AT₁ receptor inhibitor.

We also examined whether the AT₂ receptors were involved in the action of Ang II. For these studies VSMCs were pretreated with the AT₂ receptor antagonist PD 123.319 (1 μ M; 15 min) (25) before acute and prolonged Ang II treatment. AT₂ receptor inhibition decreased both acute (Fig. 1A) and prolonged (Fig. 1B) Ang II-stimulated pump activity. Taken together, the results with losartan and PD123319 indicated that Ang II regulates the Na⁺ pump in VSMCs via both AT₁ and AT₂ receptors.

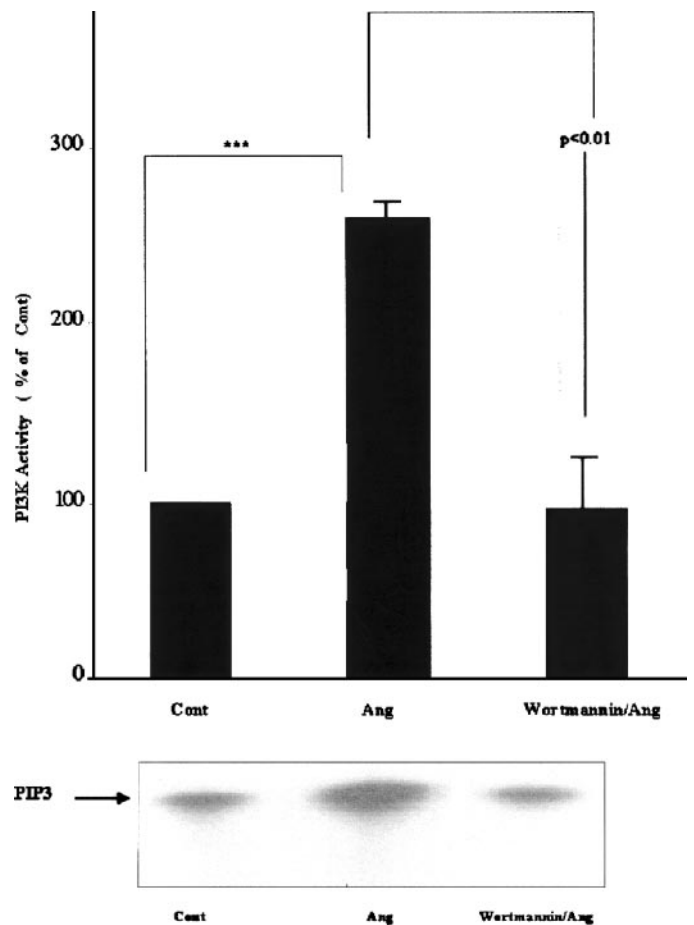


FIG. 2. Effect of Ang II on PI3K activity in VSMCs. Cells were treated with Ang II for 10 min or pretreated with wortmannin (100 nM; 30 min) and then treated with Ang II for an additional 10 min. A representative autoradiogram from TLC performed on PI3K product (phosphatidylinositol 3 phosphate) is shown at the bottom of the figure. Results are mean SEM; $n = 4$ experiments. ***, $P < 0.001$, indicates Ang *vs.* control; the other P value shown is the inhibitor-treated group *vs.* Ang II-treated cells.

Effects of Ang II on PI3K and p42/p44^{MAPK} activation

Because our results (Fig. 1, A and B) demonstrate that PI3K/p42/44^{MAPK} pathways are involved in Ang II-stimulated Na⁺ pump activity, we further investigated the effect of Ang II on stimulation of PI3K activity as well as p42/44^{MAPK} phosphorylation. Abundance of phosphorylated PI3K in antiphosphotyrosine precipitates was measured using an *in vitro* assay that quantitates phosphatidylinositol 3 phosphate (Fig. 2). A low level of PI3K activity was present in the basal state, whereas Ang II-stimulated PI3K activity (Fig. 2). However, pretreatment of cells with the PI3K inhibitor wortmannin, significantly reduced the Ang II-stimulated PI3K activity (Fig. 2). In addition, VSMCs were stimulated with Ang II (100 nM; 10 min) and the lysates were directly subjected to immunoblotting with antibody against phosphorylated p42/44^{MAPK} (Fig. 3). Ang II significantly increased the phosphorylation of p42/44^{MAPK} after 10 min (Fig. 3). However, when cells were preincubated with PD 98059, Ang II-stimulated phosphorylation of p42/p44^{MAPK} at 10 min was completely inhibited (Fig. 3). Additionally, another p42/44^{MAPK} inhibitor U0126 also decreased Ang II-stimulated phosphorylation of p42/44^{MAPK} (Fig. 3). To determine whether p42/44^{MAPK} is downstream or upstream from PI3K, VSMCs were treated with wortmannin, and p42/44^{MAPK} phosphorylation was measured. Wortmannin inhibition of PI3K did not attenuate MAPK phosphorylation

(wortmannin = 0.82, 0.22-fold, control = 1-fold, n = 5). These results suggest that PI3K and p42/44^{MAPK} activation are both involved in Ang II effects on the Na⁺ K⁺-ATPase pump.

Role of PI3K/p42/44^{MAPK} pathways in Ang II regulation of α_1 gene transcription

To further delineate the molecular mechanisms that underlie Ang II effects on the Na⁺ pump, VSMCs were transiently transfected with a luciferase reporter plasmid containing a portion of the rat Na⁺ pump subunit α_1 -promoter between -1537 to +261 bp. Exposure of cells to Ang II (100 nM; 24 h) resulted in increased luciferase activity (Fig. 4), which was completely inhibited by pretreatment with wortmannin (Fig. 4). Furthermore, p42/44^{MAPK} inhibition with PD 98059 significantly decreased Ang II-stimulated α_1 -gene transcription (Fig. 4). In addition, U0126 pretreatment also reduced Ang II-stimulated α_1 -gene transcription. These data suggest that Ang II signaling through the PI3K and MAPK pathways has a significant role in Na⁺ pump α_1 -subunit gene transcription.

To more specifically define the role of PI3K in Ang II regulation of Na⁺ pump subunit gene transcription, VSMCs were cotransfected with a Δ p85 construct, and then transfected with α_1 -promoter. These cells were then treated with Ang II for 24 h. As shown in Fig. 5, inhibition of p85 significantly decreased Ang II-mediated α_1 -gene transcription.

FIG. 3. Effect of Ang II on p42/44^{MAPK} phosphorylation in VSMCs. Cells were treated with Ang II for 10 min or pretreated with PD 98059 (50 μ M; 1 h) or U0126 (10 μ M; 15 min) and then treated with Ang II for an additional 10 min. A representative immunoblot is shown at the bottom of the figure. Results are mean \pm SEM; n = 3–4 experiments; **, $P < 0.01$, indicates Ang vs. control; the other P values shown are inhibitor-treated groups vs. Ang II-treated cells.

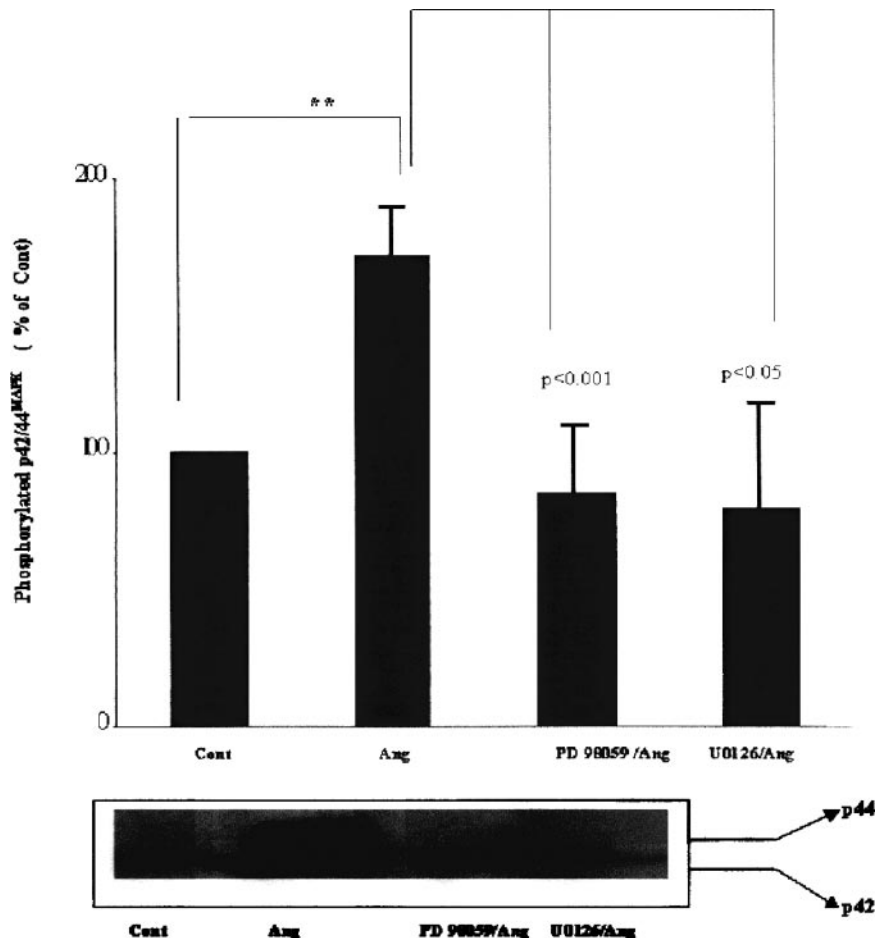
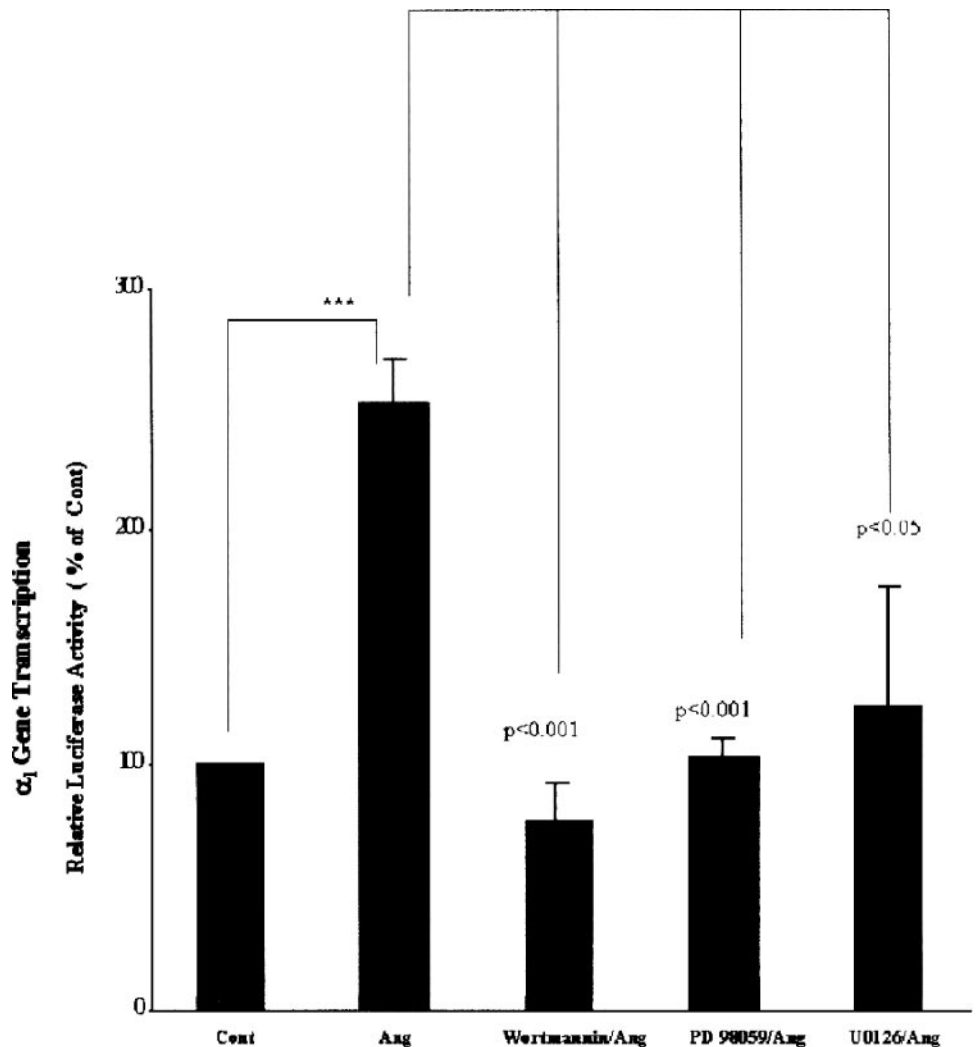


FIG. 4. Role of PI3K and p42/44^{MAPK} in Ang II-stimulated Na⁺ pump α_1 -promoter transcription. A luciferase reporter plasmid containing a portion of the Na⁺ pump α_1 -subunit promoter between -1537 to +261 bp was transiently transfected into VSMCs, and control values were assessed a relative value of 100%. Cells were treated with Ang II (100 nM; 24 h) or pretreated with wortmannin (100 nM; 30 min), PD 98059 (50 μ M; 1 h), or U0126 (10 μ M; 15 min) before Ang II treatment. Results are mean \pm SEM; n = 3–11 experiments. ***, $P < 0.001$, indicates Ang vs. control; the other P values shown are inhibitor-treated groups vs. Ang II-treated cells.



Thus, PI3K plays a significant role in Ang II regulation of α_1 -gene transcription.

Ang II-stimulated α_1 -gene transcription is blocked with amiloride and losartan in VSMCs

Because we demonstrated that Ang II-stimulated Na⁺ pump activity was suppressed by blocking either AT₁ or AT₂ receptors with either losartan or PD 123319, respectively (Fig. 1, A and B), or inhibiting Na⁺/H⁺ exchanger with amiloride (Fig. 1B), it was of interest to determine whether these inhibitors affected Ang II-stimulated α_1 -gene transcription. Amiloride as well as losartan inhibited the effect of Ang II on α_1 -subunit gene transcription (Fig. 6); however, PD 123319 treatment did not decrease Ang II-induced α_1 -promoter expression. These data suggest that Ang II effects on Na⁺ pump activity as well as α_1 -promoter transcription are mediated via AT₁ receptors, but not AT₂ receptors, and that the Na⁺/H⁺ exchanger is also involved in these effects.

Ang II effects on β_1 -gene transcription in VSMCs

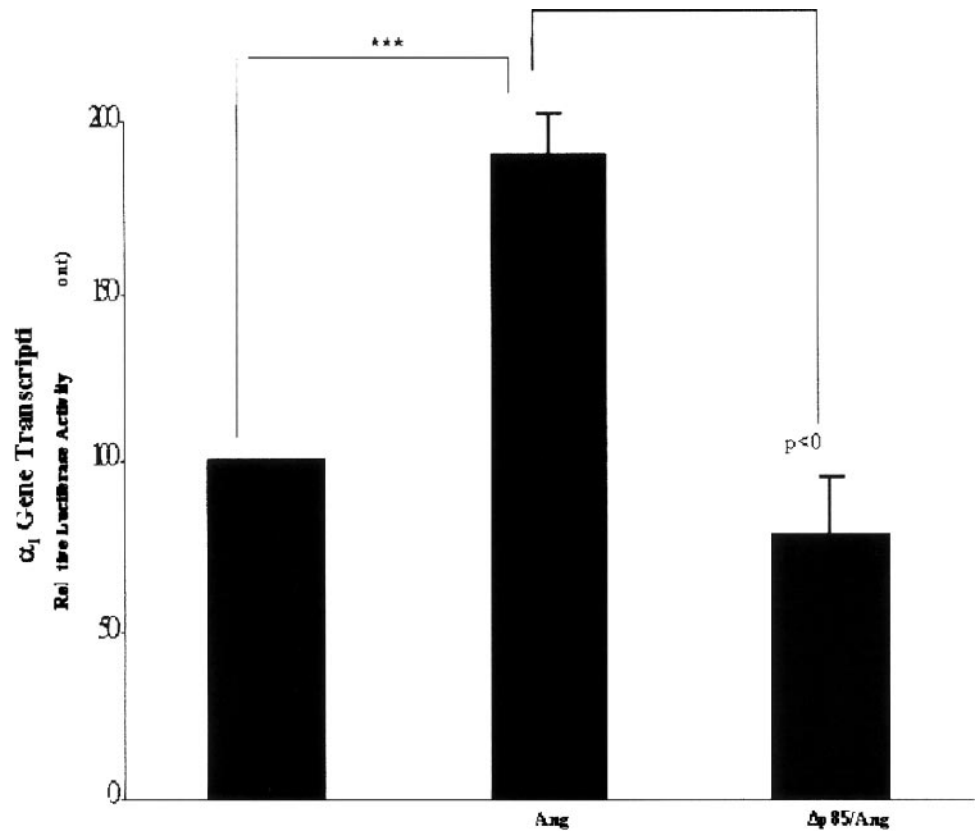
To evaluate whether Ang II stimulates Na⁺ pump β_1 -subunit gene transcription, VSMCs were transiently trans-

fectured with a luciferase reporter plasmid containing a portion of the rat β_1 -promoter between -817 to +151 bp. Exposure of cells to 100 nM Ang II for 24 h resulted in an increase in Ang II stimulated β_1 -gene transcription (Fig. 7). To examine whether PI3K and the associated signaling pathways are involved in Ang II-stimulated β_1 -gene transcription, VSMCs were pretreated with p42/44^{MAPK} inhibitor U0126 or co-transfected with Δ p85 construct. Our results demonstrate that neither PI3K nor p42/44^{MAPK} pathway is involved in Ang II-stimulated β_1 -gene transcription (U0126/Ang II = 93 \pm 13%; n = 4, Δ p85/AngII = 99.6.1% vs. control = 100%, n = 3). Thus, Ang II signaling is necessary for β_1 -subunit gene transcription; however, PI3K and p42/44^{MAPK} do not appear to be involved in its effects on β_1 -gene transcription.

Discussion

The ability of Ang II to significantly increase Na⁺ pump activity and expression has been demonstrated in several systems (5, 26), but understanding of the intracellular signal transduction pathways involved is incomplete. Our observation of rapid Ang II (10 min) stimulation of Na⁺ pump activity is consistent with previous observations that Ang II,

FIG. 5. Role of PI3K in Ang II-stimulated Na⁺ pump α_1 -promoter transcription. Before Ang II treatment, a luciferase reporter plasmid containing a portion of the Na⁺ pump α_1 -subunit promoter between -1537 and +261 bp was transiently transfected into VSMCs or VSMCs cotransfected with $\Delta p85$. Control values were assessed a relative value of 100%. After transfection, cells were treated with Ang II (100 nM; 24 h). Results are mean \pm SEM; n = 3–11 experiments. ***, $P < 0.001$, indicates Ang vs. control; the other P value shown is cotransfected vs. Ang II-treated cells.



acting via its G protein-linked receptor, also induces rapid tyrosine phosphorylation of IRS-1 (27–29) and binding of PI3K to IRS-1 in VSMCs (29). The data presented in this paper indicate that the stimulation of the VSMC Na⁺ pump by Ang II involves the PI3K and MAPK pathways because activation was blocked with pharmacological inhibition of PI3K and MAPK. With regard to the PI3K pathway, pretreatment with wortmannin or LY29404 (inhibitors of PI3K) (21) blocked the activation of the Na⁺ pump after both acute (10 min) and 24-h exposure to Ang II. To examine the regulation of Na⁺ pump in greater detail, VSMCs were transfected with $\Delta p85$ construct (lacking the binding site for the p110 catalytic subunit of PI3K). In concert with our inhibitor data, $\Delta p85$ cotransfection decreased Ang II-stimulated α_1 - but not β_1 -gene transcription.

Another important signal transduction pathway, MAPK, was also studied because it was previously shown that activation of MAPK via Ras/MEK contributes to Ang II-stimulated vascular contraction, injury, hypertrophy, and hypertension in rodents (10, 30). In the current study, Ang II-induced activation of p42/44^{MAPK} in VSMCs within 10 min. This activation is transient because incubation with Ang II for 24 h was no longer associated with activation of this kinase pathway, possibly reflecting a tachyphylaxis for this effect of Ang II.

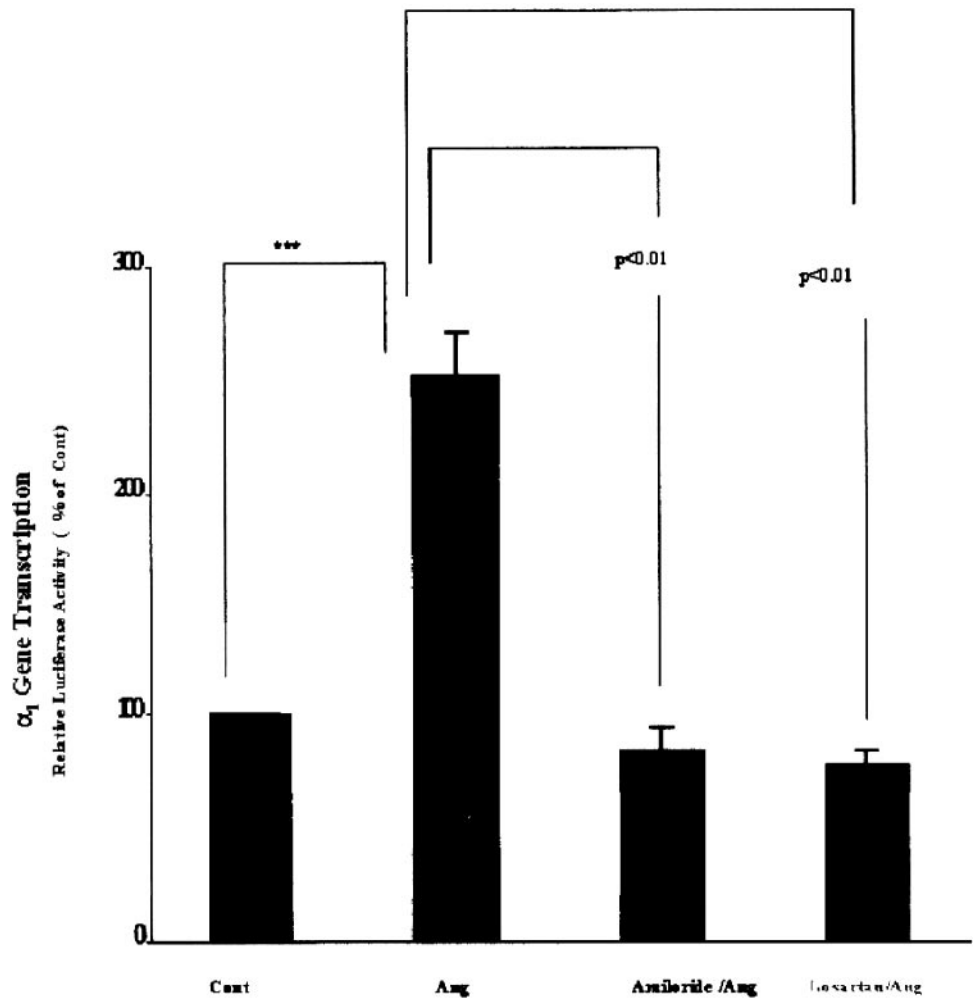
Although there is no previously reported evidence of cross-talk between the p42/44^{MAPK} and PI3K pathways in VSMCs, in the current investigation inhibition of either p42/p44^{MAPK} or PI3K activity diminished the induction of Na⁺ pump activity, suggesting that both pathways contribute to

the action(s) of Ang II. Thus, the ability of Ang II to induce activation of PI3K (Fig. 2) and p42/44^{MAPK} (Fig. 3) is consistent with the notion that Ang II activates the Na⁺ pump in VSMCs through both PI3K and p42/44^{MAPK} signaling pathways (9, 29).

The role of p42/44^{MAPK} activation in Ang II-stimulated Na⁺ pump activity was assessed, using p42/44^{MAPK} inhibitor PD 98059. This inhibitor suppressed phosphorylation of p42/44^{MAPK} in VSMCs treated with Ang II (Fig. 3). Furthermore, pretreatment of cells with PD 98059 also abolished both acute and long-term Ang II-stimulated Na⁺ pump activity. Thus, these data demonstrate that p42/44^{MAPK} signaling is necessary for the both acute and 24-h Ang II stimulation of the Na⁺ pump in VSMCs.

Ang II receptor 1 (AT₁) stimulation causes rapid but transient p42/44^{MAPK} activation via multiple signaling pathways that include protein kinase C/Ras/Raf, Pyk-2, and growth factor receptors such as epithelial growth factor (EGF) and platelet-derived growth factor (1, 31). In the rat kidney proximal convoluted tubule, EGF has been reported to stimulate Na⁺ reabsorption mediated by tyrosine phosphorylation. Activation of receptor tyrosine kinases by EGF acts on the Na⁺ pump to stimulate ouabain-sensitive ⁸⁶Rb⁺ uptake (32), suggesting that the AT₁ receptor has a role in Na⁺ pump regulation. In this study we observed that an AT₁ as well as AT₂ receptor antagonist blocked both acute and chronic stimulation of VSMC Na⁺ pump activity by Ang II. As shown in Fig. 1, both the AT₁ receptor inhibitor losartan and the AT₂ blocker PD 123.319 inhibits VSMC Na⁺ pump activity. Therefore, it can be concluded that Ang II-stimulated Na⁺ pump

FIG. 6. Effect of Na⁺/H⁺ exchanger inhibitor (amiloride) or AT₁ receptor (losartan) blocker on Ang II-induced Na⁺ pump α_1 -promoter transcription. A luciferase reporter plasmid containing a portion of the rat Na⁺ pump α_1 -subunit promoter between -1537 and +261 bp was transiently transfected into VSMCs, and control values were assessed a relative value of 100%. Cells were treated with Ang II (100 nM; 24 h) or pretreated with either amiloride (10 μ M min) or losartan (1 nM; 15 min) before Ang II treatment. Results are mean \pm SEM; n = 3–11 experiments. ***, $P < 0.001$, indicates Ang vs. control; the other P values shown are inhibitor-treated groups vs. Ang II-treated cells.



activity is mediated via both AT₁ and AT₂ receptors. In this regard, it is also of interest to note that previous studies looking at other cells types (32, 33) indicate that stimulation of AT₂ receptors may offset the AT₁ receptor-mediated actions of Ang II on Na⁺ pump activity (34, 35). When AT₁ is blocked, increased Ang II may act on AT₂ receptors (36).

The effect of the Na⁺/H⁺ exchanger on Ang II action was investigated because it has been previously suggested that an increase in Na⁺ influx is required for Ang II-stimulated Na⁺ pump activity in VSMCs (15, 24, 27, 37). A variety of growth factors and vasoconstrictors activate Na⁺/H⁺ exchange, leading to increases in intracellular Na⁺ and intracellular alkalinization, with secondary activation of the Na⁺ pump to restore homeostasis (27, 37). In the current study, however, acute Ang II stimulation of ⁸⁶Rb flux in VSMCs was not inhibited by amiloride, a relatively specific inhibitor of Na⁺/H⁺ exchange (37, 38), suggesting that this acute stimulation was not dependent on Na⁺/H⁺ activation. By contrast, amiloride inhibits the longer-term (24 h) effect of Ang II on Na⁺ pump activity, demonstrating the importance of increased Na⁺/H⁺ exchange activity and the associated increase in intracellular Na⁺. In this regard, an increase in Na⁺ influx may have a major role in the mechanism of Ang II-stimulated up-regulation of Na⁺ pump α_1 promoter tran-

scription. Increased Na⁺ influx may serve as a signal either directly, or indirectly, to up-regulate α_1 -subunit gene transcription. Consistent with this notion, increased intracellular Na⁺ has been shown to stimulate gene transcription of the Na⁺ pump α_1 -isoform in VSMCs (39). Furthermore, it has been reported that treatment of VSMCs with monensin can increase intracellular Na⁺, inducing a dose-dependent up-regulation of Na⁺ pump α_1 -, α_2 -, and β_1 -subunit mRNA levels (40). Investigators have characterized a transcriptional Na⁺-response mechanism, defining a positive Na⁺-response regulatory region in the α_1 - and α_2 -genes of the Na⁺ pump, and they detected a Na⁺-response nuclear DNA binding protein (40). It remains to be resolved how increased intracellular Na⁺ stimulates Na⁺ pump α_1 -, α_2 -, and β_1 -subunit gene transcription.

Transcriptional/translational regulation of Na⁺ pump α_1 - and β_1 -subunit isoforms has been reported to be altered in several disease states and also altered in a number of tissues in response to various agonists (15, 39–41). Although it has been previously shown that Ang II increases α_1 - and β_1 -mRNA content in VSMCs (15, 16, 38), the molecular basis of the increase of mRNA levels had not been delineated. In the present investigation, an increase in α_1 - and β_1 -gene transcription was detected 24 h after Ang II treatment of the

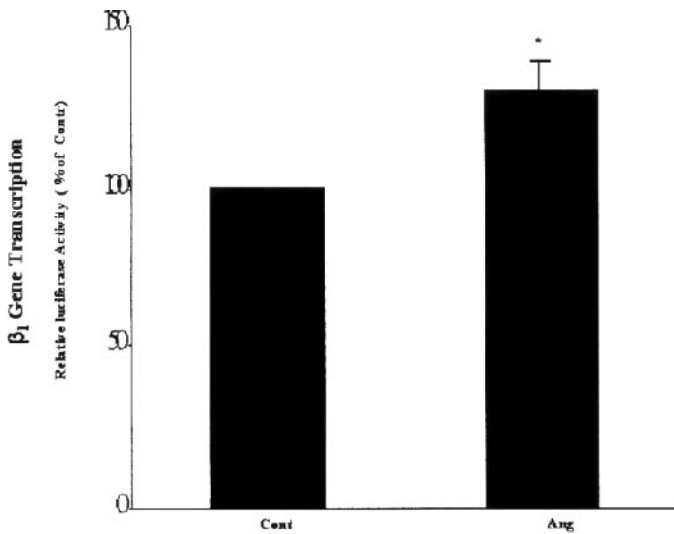


FIG. 7. Effect of Ang II treatment on Na⁺ pump β_1 -promoter transcription. A luciferase reporter plasmid containing a portion of the rat Na⁺ pump β_1 -subunit promoter between -817 and +151 bp was transiently transfected into VSMCs. Control values were assessed a relative value of 100%. Cells were treated with Ang II (100 nM; 24 h). Results are mean \pm SEM; n = 3 experiments. *, $P < 0.05$, indicates Ang vs. control.

VSMCs. To our knowledge, this is the first report that Ang II stimulates both α_1 - and β_1 -gene transcription in VSMCs. The Ang II-stimulated increase in α_1 -gene transcription was inhibited by wortmannin, U0126, and PD 98059 as well as in cotransfection with $\Delta p85$, suggesting that this Ang II effect is mediated through both PI3K and p42/44^{MAPK} signaling. Furthermore, Ang II stimulation of α_1 -gene transcription was inhibited by losartan and amiloride, implicating the AT₁ receptor and Na⁺/H⁺ exchange in this process. Our results also show that Ang II-stimulated β_1 -gene transcription is not mediated via PI3K/p42/44^{MAPK}. Thus, the evaluation of potential signaling pathways having a role in Ang II regulation of β_1 -subunit should be a fruitful area of future investigation.

In summary, results of this investigation indicate that Ang II-stimulated Na⁺ pump activity in VSMCs via binding to AT₁/AT₂ receptors initiates both PI3K and p42/44^{MAPK} signal transduction pathways, leading to up-regulation of gene transcription of the α_1 -catalytic subunit. This effect is observed rapidly and lasts for at least 24 h. Na⁺/H⁺ exchange is also involved in the sustained effects of Ang II on Na⁺ pump activity. The Na⁺ pump is an important mediator of vascular volume, tone intracellular [Ca²⁺], intracellular pH, and growth (42), and abnormal regulation of the Na⁺ pump has been implicated in a number of disease states such as hypertension, diabetes, and arteriosclerosis (1, 2, 24). Because Ang II is a tissue-produced autocrine/paracrine factor, local regulation of the Na⁺ pump by Ang II is of potential physiological significance. In conclusion, our results suggest that regulation of Na⁺ pump activity and gene transcription is mediated via both PI3K and p42/44^{MAPK} pathways.

Acknowledgments

Received January 21, 2003. Accepted November 11, 2003.

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This work was supported by grants from the NIH (RO1-HL-63904-01), the Veterans Affairs Merit System (0018), and the American Diabetes Association (RA0095).

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