Increased AQP2 targeting in primary cultured IMCD cells in response to angiotensin II through AT₁ receptor

Yu-Jung Lee,¹ In-Kyung Song,¹ Kyung-Jin Jang,¹ Jakob Nielsen,² Jørgen Frøkiaer,²,³ Søren Nielsen,² and Tae-Hwan Kwon¹,²

¹Department of Biochemistry and Cell Biology, School of Medicine, Kyungpook National University, Taegu, Korea; ²Water and Salt Research Center, University of Aarhus, and ³Institute of Clinical Medicine, Aarhus University Hospital, Aarhus, Denmark

Submitted 19 March 2006; accepted in final form 31 July 2006

Lee YJ, Song IK, Jang KJ, Nielsen J, Frøkiaer J, Nielsen S, Kwon TH. Increased AQP2 targeting in primary cultured IMCD cells in response to angiotensin II through AT₁ receptor. Am J Physiol Renal Physiol 292: F340–F350, 2007.—Vasopressin and angiotensin II (ANG II) play a major role in renal water and Na⁺ reabsorption. We previously demonstrated that ANG II AT₁ receptor blockade decreases dDAVP-induced water reabsorption and AQP2 levels in rats, suggesting cross talk between these two peptide hormones (Am J Physiol Renal Physiol 288: F673–F684, 2005). To directly address this issue, primary cultured inner medullary collecting duct (IMCD) cells from male Sprague-Dawley rats were treated for 15 min with (1) vehicle, (2) ANG II, (3) ANG II + the AT₁ receptor blocker candesartan, (4) dDAVP, (5) ANG II + dDAVP, or (6) ANG II + dDAVP + candesartan. Immunofluorescence microscopy revealed that 10⁻⁸ M ANG II or 10⁻¹¹ M dDAVP (protocol 1) was associated with increased AQP2 labeling of the plasma membrane and decreased cytoplasmic labeling, respectively. cAMP levels increased significantly in response to 10⁻⁸ M ANG II and were potentiated by cotreatment with 10⁻¹¹ M dDAVP. Consistent with this finding, immunoblotting revealed that this cotreatment significantly increased expression of phosphorylated AQP2. ANG II-induced AQP2 targeting was blocked by 10⁻⁸ M candesartan. In protocol 2, treatment with a lower concentration of dDAVP (10⁻¹² M) or ANG II (10⁻⁹ M) did not change subcellular AQP2 distribution, whereas 10⁻¹² M dDAVP + 10⁻⁹ M ANG II enhanced AQP2 targeting. This effect was inhibited by cotreatment with 10⁻⁸ M candesartan. ANG II-induced cAMP accumulation and AQP2 targeting were inhibited by inhibition of PKC activity. In conclusion, ANG II plays a role in the regulation of AQP2 targeting to the plasma membrane in IMCD cells through AT₁ receptor activation and potentiates the effect of dDAVP on AQP2 plasma membrane targeting.

aquaporin; cAMP; intracellular trafficking; dDAVP; protein kinase C

RENAL REABSORPTION AND EXCRETION of water and Na⁺ are critical to the regulation of extracellular fluid volume. Vasopressin and angiotensin II (ANG II) have important roles in the renal conservation of water and Na⁺ (13, 26, 25, 38). Vasopressin is a peptide hormone that regulates renal blood flow, glomerular filtration rate, and aldosterone secretion. Moreover, it is well established that ANG II increases Na⁺ and bicarbonate reabsorption in the kidney proximal tubule via the AT₁ receptor (14). Recent studies have demonstrated that ANG II also has an important effect on the thick ascending limb (TAL) and collecting duct, where ANG II receptor mRNA and protein are present (15, 47). For example, 1) ANG II stimulates the activity of the epithelial Na⁺ channel in an isolated cortical collecting duct (41), and ANG II induces vasopressin V₂ receptor mRNA expression in cultured IMCDs (53), 2) ANG II infusion in rats increases expression of the Na⁺/H⁺ exchanger NHE3 and the Na⁺-K⁺-2Cl⁻ cotransporter NKCC2 in the medullary TAL (mTAL) (27), and 3) ANG II increases vasopressin-stimulated urea transport in the rat terminal IMCD (21). These findings suggest an important role of ANG II in regulation of urinary concentration capacity.

The action of vasopressin and ANG II is mediated by intracellular secondary messengers, which are mainly coupled to the cAMP-PKA and phosphoinositide pathways, respectively. Vasopressin induces an increase in intracellular cAMP levels (10, 23), whereas ANG II induces a rise in intracellular Ca²⁺ concentration ([Ca²⁺]i) by inositol 1,4,5-triphosphate (4) and protein kinase C (PKC) activation by diacylglycerol (20). Importantly, it has previously been demonstrated that ANG II potentiates the vasopressin-dependent cAMP accumulation in Chinese hamster ovary cells transfected with cDNA of AT₁A and V₂ receptors (24). Moreover, forskolin potentiates the ANG II-induced increase in [Ca²⁺]i in an isolated cortical TAL (19). These results suggest cross talk between the signaling pathways leading to ANG II and vasopressin action.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: T.-H. Kwon, Dept. of Biochemistry and Cell Biology, School of Medicine, Kyungpook National Univ., Dongin-dong 101, Taegu 700-422, Korea (e-mail: thkwon@knu.ac.kr).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

First published August 8, 2006; doi:10.1152/ajprenal.00090.2006.
pathways of vasopressin and ANG II. Consistent with these findings, we recently demonstrated that pharmacological blockade of the ANG II AT1 receptor in dDAVP-treated rats subjected to dietary NaCl restriction (to induce high plasma endogenous ANG II levels) increased urine production, decreased urine osmolality, and blunted the dDAVP-induced upregulation of AQP2 and phosphorylated AQP2 (i.e., AQP2 phosphorylated in the PKA-phosphorylation consensus site Ser256) expression in the inner medulla (28).

Since regulation of intracellular AQP2 trafficking and osmotic water permeability in the collecting duct principal cells involves intracellular cAMP production (11, 45), we hypothesized that these two peptide hormones have additive effects on the intracellular cAMP accumulation in IMCD cells and, hence, on the stimulation of AQP2 phosphorylation, AQP2 trafficking, and the increase in osmotic water permeability of the collecting duct cells. To directly address this issue, the aim of the present study was to examine the direct effect of 1) dDAVP or ANG II alone and 2) dDAVP + ANG II in primary cultured IMCD cells. We also examined the effect of dDAVP or ANG II alone in AQP2-transfected Madin-Darby canine kidney (MDCK) cells.

Specifically, we examined 1) the short-term effect of dDAVP or ANG II on AQP2 targeting to the plasma membrane, corresponding changes of cAMP level and phosphorylated AQP2 expression, 2) the additive effect of ANG II in potentiating the effect of dDAVP on AQP2 targeting to the plasma membrane, cAMP level, and phosphorylated AQP2 expression (i.e., dDAVP + ANG II), 3) whether the effect of ANG II on AQP2 targeting was mediated by the ANG II AT1 receptor, and 4) whether ANG II-induced activation of PKC activity played a role in the effect of ANG II on AQP2 targeting and cAMP levels.

METHODS

Primary Culture of Rat Kidney IMCD Cells: Protocols 1, 2, and 3

Primary cultures enriched in IMCD cells were prepared from pathogen-free male Sprague-Dawley rats (200–270 g body wt; Samtako, Osan, Korea) as described previously (6). The animal protocols were approved by the Animal Care and Use Committee of the Kyungpook National University, Korea. Both kidneys were rapidly removed from male Sprague-Dawley rats under light enflurane inhalation anesthesia. The kidneys were placed in cold D-PBS [1,000 ml of double-distilled H2O, 0.2 g of KCl, 0.2 g of KH2PO4, 1.15 g of Na2HPO4, 80 mM urea, and 130 mM NaCl (pH 7.4, 640 mosmol/kgH2O)] and quickly dissected into inner medulla and other parts. The inner medulla was placed on an ice-cold petri dish, minced, transferred to a 25-cm2 tissue culture flask (catalog no. 1088815, Roche, Mannheim, Germany), 7 mg of collagenase B (catalog no. 1088815, Roche, Mannheim, Germany), 5 mg of hyaluronidase (catalog no. H-3884, Sigma, St. Louis, MO), 80 mM urea, and 130 mM NaCl, and incubated at 37°C under continuous agitation (300 rpm) for 90 min in a humidified incubator under 5% CO2-95% air. Centrifugation of the resulting suspension at 160 g for 1 min yielded a pellet that contained IMCD fragment and IMCD cells. The pellet was washed in prewarmed culture medium without enzyme [DMEM-Ham’s F-12 without phenol red, 80 mM urea, 130 mM NaCl, 10 mM HEPES, 2 mM l-glutamine, penicillin-streptomycin (10,000 U/ml), 50 mM hydrocortisone, 5 mM 3,3,5-triiodothyronine, 1 mM sodium selenate, 5 mg/l transferrin, and 10% FBS (pH 7.4, 640 mosmol/kgH2O)]. The IMCD cell suspension was then seeded in human fibronectin-coated chamber slides (Lab-Tek Chamber Slides System, catalog no. 177402, NUNC, Roskilde, Denmark) for immunocytochemical analysis or in a 24-well chamber (catalog no. 142485, NUNC) for immunoblot analysis and cAMP measurement. For immunoblot analysis, an IMCD cell suspension of only the lower two-thirds of the inner medulla (IM-2 and IM-3) was cultured to exclude the cell component of intercalated cells expressed in IM-1. IMCD cells were fed every 24 h and grown in hypertonic culture medium (640 mosmol/kgH2O) supplemented with 10% FBS at 37°C in 5% CO2-95% air for 3 days and then in FBS-free culture medium for an additional 1 day before the experiment on day 5.

Experimental Protocols for Primary Cultured IMCD Cells

Protocol 1: higher dose of dDAVP and ANG II. IMCD cells were treated with 1) vehicle (FBS-free culture medium), 2) dDAVP (10−11 M), 3) ANG II (10−8 M), 4) dDAVP (10−11 M) + ANG II (10−8 M), 5) ANG II (10−8 M) + AT1 receptor blocker (candesartan, 10−5 M; a gift from AstraZeneca Pharmaceutical), and 6) dDAVP (10−11 M) + ANG II (10−8 M) + candesartan (10−5 M) for 15 min.

Protocol 2: lower dose of dDAVP and ANG II. IMCD cells were treated with 1) vehicle (FBS-free culture medium), 2) dDAVP (10−12 M), 3) ANG II (10−9 M), 4) dDAVP (10−12 M) + ANG II (10−9 M), 5) ANG II (10−9 M) + candesartan (10−5 M), and 6) dDAVP (10−12 M) + ANG II (10−9 M) + candesartan (10−5 M) for 15 min.

Protocol 3: ANG II with or without PKC inhibitor. IMCD cells were treated with 1) vehicle (FBS-free culture medium), 2) vehicle (FBS-free culture medium) + the PKC inhibitor staurosorpine (catalog no. S6942, Sigma; 10−7 M), 3) ANG II (10−8 M), and 4) ANG II (10−8 M) + staurosorpine (10−7 M) for 15 min. The cells subjected to PKC inhibition were preincubated with staurosorpine (10−7 M) for 10 min and then incubated with vehicle or ANG II for another 15 min in the presence of staurosorpine.

Primary Culture of Rat Kidney IMCD Cells in Isosmotic or Hyperosmotic Culture Medium Without Urea

To avoid the potential effects of urea or hyperosmolality on the short-term AQP2 targeting in the primary cultured IMCD cells (8, 16, 50), control experiments were done with primary cultured IMCD cells grown in hyperosmotic (640 mosmol/kgH2O) and isosmotic (300 mosmol/kgH2O) culture media, neither of which contained urea. The osmolality of the culture medium [DMEM-Ham’s F-12 without phenol red, 10 mM HEPES, 2 mM l-glutamine, penicillin-streptomycin (10,000 U/ml), 30 mM hydrocortisone, 5 mM 3,3,5-triiodothyronine, 1 mM sodium selenate, 5 mg/l transferrin, and NaCl (pH 7.4)] was adjusted by changing NaCl concentration and determined by freezing-point depression (Osmomat 030-D, Gonotec, Berlin, Germany). IMCD cells were fed every 24 h and cultured in medium that was supplemented with 10% FBS at 37°C 5% CO2-95% air for 3 days and then in FBS-free culture medium for additional 1 day before the experiment on day 5. IMCD cells cultured in hyperosmotic culture medium without urea (640 mosmol/kgH2O) were treated with 1) vehicle, 2) dDAVP (10−11 M), or 3) ANG II (10−8 M) for 15 min on day 5.

Immunocytochemistry of Rat Kidney IMCD Cells

IMCD cells were grown to confluence in each chamber for 4 days and treated on day 5 after seeding according to the experimental protocols. After treatment, cells in each chamber were washed twice in PBS and fixed with 4% paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature. After the cells were washed twice in PBS, they were permeabilized with 0.3% Triton X-100 in PBS at room temperature for 15 min. The cells were incubated with anti-AQP2 antibody (36) in PBS overnight at 4°C, washed in PBS, and incubated with goat anti-rabbit IgG Alexa Fluor 488 secondary antibody (Molecular Probes, Eugene, OR) for 1.5 h at room temperature. Cells were washed and mounted with a hydrophilic mounting medium containing....
antifading reagent (catalog no. P36930, Molecular Probes). Light microscopy was carried out with a light microscope (Axioplan2 imaging, Zeiss, Jena, Germany) or an inverted microscope (model DM IRB, Leica Microsystems, Wetzlar, Germany) equipped with a CoolSNAP HQ camera (Photometrics, Tucson, AZ).

cAMP Measurement in Primary Cultured IMCD Cells

IMCD cells were cultured to confluence in a 24-well chamber for 4 days after they were seeded and treated on day 5 according to the protocols. All measurements was carried out in the presence of 1 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma) to inhibit cyclic nucleotide phosphodiesterases. After 10 min of preincubation with 1 mM IBMX, dDAVP, ANG II, or dDAVP + ANG II was added for an additional 15 min in the continued presence of IBMX. cAMP content of the samples was measured using a competitive enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI), and the results were expressed in picomoles per milliliter of cell lysate (2 × 10^5 cells seeded per chamber). Each determination was performed in triplicate. For the measurement of cAMP levels in response to PKC inhibitor treatment (protocol 3), we repeated the determination twice. Each determination was performed in triplicate; thus the results were pooled and expressed as a fraction of the control level (vehicle-treated group).

Semiquantitative Immunoblotting of Phosphorylated AQP2

After incubation, IMCD cells were homogenized in RIPA buffer [10 mM Tris-HCl, 0.15 M NaCl, 1% NP-40, 1% Na-deoxycholate, 0.5% SDS, 0.02% sodium azide, and 1 mM EDTA (pH 7.4)]. The total protein concentration was measured using the bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL). All samples were adjusted with isolation solution to the identical final protein concentrations, solubilized at 65°C for 15 min in Laemml sample buffer, and then stored at 4°C. For confirmation of equal loading of protein, an initial gel was stained with Coomassie blue dye as described previously (28, 36). SDS-PAGE was performed on 12% polyacrylamide gels. The proteins were transferred from the gel electrophoretically (Mini Protein II, Bio-Rad) to nitrocellulose membranes (Hybond ECL RPN3032D, Amersham Pharmacia Biotech, Little Chalfont, UK). After transfer, the blots were blocked with 5% milk in PBS-T (80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, and 0.1% Tween 20 (pH 7.5)) for 1 h and incubated overnight at 4°C with antibodies directed against phosphorylated AQP2 (7). The sites of antibody-antigen reaction were visualized with horseradish peroxidase-conjugated secondary antibodies (1:3,000 dilution; catalog no. P-448, Dako, Glostrup, Denmark) with an enhanced chemiluminescence (ECL) system and exposed to photographic film (Hyperfilm ECL, RPN3103K, Amersham Pharmacia Biotech). For quantification of the band densities (29- and 35- to 50-kDa phosphorylated AQP2 bands), the films were scanned and the densitometry values were normalized to facilitate comparisons. For immunoblot analysis, in each group, four different protein samples were obtained from IMCD cells cultured in four different culture well chambers. For each immunoblotting gel, two different protein samples from each group were loaded. The measured band densities (i.e., from 4 different protein samples in each group) were pooled and expressed as a fraction of the control level (vehicle-treated group).

AQP2-Transfected MDCK Cells

pCDNAI/Neo with rat cDNA encoding AQP2 (tagged with a COOH-terminal c-myc epitope) was generously provided by Dr. D. Brown (30). MDCK cells were grown in DMEM supplemented with 10% (vol/vol) FBS at 37°C in 5% CO₂. MDCK cells were then seeded in chamber slides (Lab-Tek Chamber Slides System) and transiently transfected using Lipofectamine reagent (catalog no. 18324-020, Invitrogen). Cells were kept in serum-free medium containing 10 μM indomethacin overnight before experiments to inhibit endogenous prostaglandin production and to prevent cAMP production and analyzed 48 h after transfection (35). Cells were treated with vehicle or 10⁻⁸ M dDAVP or 10⁻⁷ M ANG II for 15 min. The experiment was repeated three times. Cells were fixed in 4% paraformaldehyde for 10 min, rinsed twice in PBS, and blocked for 15 min in blocking/ permeabilization solution (PBS containing 0.1% BSA and 0.3% Triton X-100). Cells were incubated with anti-c-myc antibody (catalog no. C-3956, Sigma) diluted in blocking solution overnight at 4°C, washed three times in PBS, and incubated with the secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes; 1:400 dilution) for 1 h at room temperature. Cells were analyzed using an inverted microscope (model DM IRB, Leica Microsystems) equipped with a laser confocal scanning unit (model CSU-10, Yokokawa, Tokyo, Japan), an Ar-Kr laser (Melles Griot, Irvine, CA), and a CoolSNAP HQ camera. Fluorescent images were acquired, and semi-quantitative measurement of average pixel intensities in the plasma membrane and cytoplasmic AQP2 labeling was done by using Metamorph software (Molecular Devices, Sunnyvale, CA). The average pixel intensities of the plasma membrane AQP2 labeling were expressed as a fraction of that of cytoplasmic AQP2 labeling.

Presentation of Data and Statistical Analyses

Values are means ± SE. Data were analyzed by one-way ANOVA followed by Tukey’s honestly significant different multiple-comparisons test. Multiple-comparisons tests were applied only when a significant difference was determined by ANOVA (P < 0.05). P < 0.05 was considered statistically significant.

RESULTS

Increased AQP2 Targeting to the Plasma Membrane in Response to Short-Term Treatment With dDAVP or ANG II: Protocol 1

AQP2 immunofluorescent labeling was largely dispersed along the cytoplasm of primary cultured IMCD cells in the absence of dDAVP or ANG II stimulation (Fig. 1A). In contrast, treatment with 10⁻¹¹ M dDAVP for 15 min was associated with an increase in AQP2 immunofluorescent labeling of the plasma membrane and a decrease in labeling in the cytoplasm (Fig. 1C), indicating that dDAVP increased AQP2 targeting to the plasma membrane in IMCD cells. Treatment with 10⁻⁸ M ANG II for 15 min was also associated with enhanced AQP2 labeling of the plasma membrane, indicating that ANG II per se directly stimulated AQP2 targeting to the plasma membrane of IMCD cells (Fig. 1E). Pretreatment with 10⁻⁵ M candesartan, an ANG II AT₁ receptor blocker, inhibited AQP2 targeting to the plasma membrane induced by 10⁻⁸ M ANG II (Fig. 1F), demonstrating that the ANG II-induced AQP2 targeting was mediated by the ANG II AT₁ receptor. Treatment with 10⁻¹¹ M dDAVP + 10⁻⁸ M ANG II was also associated with strong AQP2 labeling of the plasma membrane (Fig. 1B), similar to the effect of 10⁻¹¹ M dDAVP (Fig. 1C) or 10⁻⁸ M ANG II (Fig. 1E), and the effect was not completely inhibited by pretreatment with 10⁻⁵ M candesartan (Fig. 1D).

To support the finding that dDAVP- or ANG II-stimulated AQP2 targeting to the plasma membrane was specific, we conducted two control experiments to show that the effects are independent of the urea (80 mM) in culture medium and the hyperosmolality (640 mosmol/kgH₂O) of the culture medium (Fig. 2). First, urea may affect PKC-mediated signaling (8), which in turn may affect regulation of AQP2 targeting. We therefore examined the effect of dDAVP and ANG II without
urea in the culture medium (Fig. 2). AQP2 immunofluorescent labeling in IMCD cells grown in culture medium without urea was dispersed in the cytoplasm in the absence of dDAVP or ANG II stimulation (Fig. 2B). Treatment for 15 min with $10^{-11}$ M dDAVP (Fig. 2C) or $10^{-8}$ M ANG II (Fig. 2D) was associated with enhanced AQP2 targeting to the plasma membrane, which indicates that the dDAVP- or ANG II-stimulated AQP2 targeting (Figs. 1 and 3) was not dependent on urea. Second, hyperosmolality can regulate AQP2 in a vasopressin-independent manner (50). Because the hyperosmolar (640 mosmol/kgH$_2$O) culture medium used in the first study might have affected the results, we examined AQP2 labeling in primary cultured IMCD cells grown for 4 days in isosmolar (300 mosmol/kgH$_2$O) culture medium without urea or in hyperosmolar (640 mosmol/kgH$_2$O) culture medium without urea. AQP2 labeling was much weaker in IMCD cells cultured in isosmolar medium (Fig. 2A) than in those cultured in hyperosmolar medium (Fig. 2B). This is consistent with the previous finding that the expression of AQP2 (nonglycosylated form) was increased by hyperosmolality (50). However, immunolabeling was mainly seen in the cytoplasm in both conditions (Fig. 2, A and B), indicating that hyperosmolality of cell culture medium per se is unlikely to play a major role in the shorter-term AQP2 targeting.

Additive Effect of dDAVP and ANG II on AQP2 Targeting to the Plasma Membrane: Protocol 2

Next we tested the effects of lower doses of dDAVP and ANG II (protocol 2) to examine whether dDAVP and ANG II have an additive effect on AQP2 targeting. In the absence of dDAVP or ANG II stimulation, AQP2 labeling was dispersed along the cytoplasm of primary cultured IMCD cells (Fig. 3A), as in protocol 1 (Fig. 1A). In protocol 2, however, little AQP2 targeting to the plasma membrane was observed in response to 15 min of treatment with $10^{-12}$ M dDAVP or $10^{-9}$ M ANG II (Fig. 3, C and E). In contrast, 15 min of treatment with $10^{-12}$ M dDAVP + $10^{-9}$ M ANG II was associated with enhanced AQP2 targeting to the plasma membrane (Fig. 3B), which was blocked by cotreatment with the ANG II AT$_1$ receptor blocker.

Fig. 1. Immunofluorescence microscopy of aquaporin 2 (AQP2) in primary cultured inner medullary collecting duct (IMCD) cells (protocol 1). A: AQP2 labeling exclusively localized at the cytoplasm in response to vehicle. C and E: increase in AQP2 targeting to the plasma membrane in response to dDAVP (C) or ANG II (E). F: blockade of ANG II-induced AQP2 targeting by cotreatment with candesartan. B and D: abundant AQP2 targeting to the plasma membrane in response to dDAVP + ANG II and no inhibition by ANG II AT$_1$ receptor blockade.
candesartan (10^{-5} M; Fig. 3D). This finding strongly suggests that dDAVP and ANG II have an additive effect on AQP2 targeting to the plasma membrane of IMCD cells and that the ANG II effect is mediated by the ANG II AT1 receptor.

**cAMP Production Was Potentiated in Response to dDAVP + ANG II Compared With dDAVP or ANG II**

To examine whether the enhanced AQP2 targeting to the plasma membrane in response to dDAVP or ANG II or dDAVP + ANG II was associated with an increase in cAMP production, we measured cAMP levels in primary cultured IMCD cells. In a preliminary study to test the validity of cAMP measurement in this study, we examined the effects of 15 min of treatment with different concentrations of dDAVP (10^{-11}, 10^{-9}, and 10^{-7} M) and forskolin (10^{-4} M) on cAMP levels in primary cultured IMCD cells. Consistent with previous results (9), cAMP levels increased in response to dDAVP treatment in a dose-dependent manner (Fig. 4). Moreover, forskolin markedly increased cAMP levels in primary cultured IMCD cells (Fig. 4).

In protocol 1, cAMP levels were unchanged in response to 15 min of treatment with 10^{-11} M dDAVP, whereas levels increased in response to 10^{-8} M ANG II (Fig. 5A). Importantly, the cAMP increase after ANG II was potentiated by 10^{-11} M dDAVP + 10^{-8} M ANG II (Fig. 5A), suggesting an additive effect. In protocol 2, the changes in cAMP levels were similar to those in protocol 1 (Fig. 5B). cAMP levels were unchanged by 10^{-12} M dDAVP but increased by 10^{-9} M ANG II (Fig. 5B). The additive effect of 10^{-12} M dDAVP + 10^{-9} M ANG II on cAMP levels compared with the effect of 10^{-9} M ANG II alone did not reach statistical significance in protocol 2 (Fig. 5B; P = 0.06).

**Phosphorylated AQP2 Expression Was Increased in Response to dDAVP + ANG II**

Since cAMP activates PKA, which phosphorylates various proteins, including AQP2, we examined the changes in expression of phosphorylated AQP2 (phosphorylated in the PKA phosphorylation consensus site Ser^{256}). Treatment with 10^{-11} M dDAVP + 10^{-8} M ANG II significantly increased phosphorylated AQP2 expression to 135 ± 4% of vehicle-treated control levels (100 ± 3%, P < 0.05; Fig. 6). Treatment with 10^{-11} M dDAVP or 10^{-8} M ANG II did not significantly change phosphorylated AQP2 expression (97 ± 1% and 117 ± 2% of vehicle-treated control levels, respectively, P = not significant). This result suggests an additive effect of dDAVP + ANG II on AQP2 phosphorylation, consistent with the additive effect on cAMP production (Fig. 5) and AQP2 targeting to the plasma membrane (Fig. 3B). The increased expression of phosphorylated AQP2 by dDAVP + ANG II was attenuated by the ANG II AT1 receptor blocker candesartan (114 ± 1% of vehicle-treated control levels, P = not significant; Fig. 6). Again, this suggests that the effect of ANG II on AQP2 phosphorylation is mediated by the ANG II AT1 receptor.

**AQP2 Targeting to the Plasma Membrane Was Increased in Response to dDAVP or ANG II in AQP2-c-myc-Transfected MDCK Cells**

To confirm the results observed in primary cultured IMCD cells, we tested the direct effect of dDAVP or ANG II on subcellular AQP2 localization in MDCK cells that were transiently transfected with AQP2. In nontreated MDCK cells, AQP2 was mainly localized intracellularly [Fig. 7A; semiquantitative measurement of average pixel intensity of AQP2 label-
ing in the plasma membrane of 30 randomly selected transfected cells: 14 ± 2% of the cytoplasmic intensity (Fig. 7D). AQP2 targeting to the plasma membrane was clearly observed in cells treated for 15 min with 10⁻⁸ M dDAVP [Fig. 7B; average pixel intensity of AQP2 labeling in the plasma membrane of 30 randomly selected transfected cells: 31 ± 3% of the cytoplasmic intensity (Fig. 7E)]. Stimulation with 10⁻⁷ M ANG II also resulted in translocation of AQP2 to the plasma membrane [Fig. 7C; average pixel intensity of AQP2 labeling in the plasma membrane of 30 randomly selected transfected cells: 24 ± 2% of the cytoplasmic intensity (Fig. 7F)], supporting the results obtained in the primary cultured IMCD cells.

Treatment With PKC Inhibitor Decreased ANG II-Induced AQP2 Targeting to the Plasma Membrane and cAMP Production in Primary Cultured IMCD Cells

PKC is a component of the signal transduction pathway of ANG II. Since there is evidence demonstrating that PKC plays a role in stimulation of the receptor-coupled adenylate cyclase in some cell systems (24, 33, 43), we examined the effect of the PKC inhibitor staurosporine on ANG II-induced AQP2 targeting and cAMP production in primary cultured IMCD cells.

Fig. 3. Immunofluorescence microscopy of AQP2 in primary cultured IMCD cells (protocol 2). A: AQP2 labeling exclusively localized at the cytoplasm in response to vehicle treatment. C and E: no or little AQP2 targeting to the plasma membrane in response to dDAVP (C) or ANG II (E). B and D: increase in AQP2 targeting to the plasma membrane in response to dDAVP + ANG II and inhibition by ANG II AT1 receptor blockade.
Consistent with the results shown in Fig. 1, treatment with $10^{-8}$ M ANG II for 15 min was associated with AQP2 targeting to the plasma membrane in IMCD cells (Fig. 8A). In contrast, $10^{-8}$ M ANG II + $10^{-7}$ M staurosporine was associated with decreased AQP2 targeting (Fig. 8B). Moreover, cAMP levels increased in response to $10^{-8}$ M ANG II, whereas cAMP levels did not increase in response to $10^{-8}$ M ANG II + $10^{-7}$ M staurosporine (Fig. 9).

**DISCUSSION**

In the present study, we demonstrated that 1) short-term treatment with dDAVP or ANG II had a stimulatory effect on AQP2 targeting to the plasma membrane in primary cultured IMCD cells and AQP2-transfected MDCK cells; 2) dDAVP + ANG II potentiated cAMP production, phosphorylated AQP2 expression, and AQP2 targeting in primary cultured IMCD cells compared with dDAVP or ANG II alone; 3) the effect of ANG II on phosphorylated AQP2 expression and AQP2 targeting was inhibited by ANG II AT$_1$ receptor blockade, suggesting that the ANG II effect was mediated by the ANG II AT$_1$ receptor; and 4) ANG II + PKC inhibitor decreased ANG II-induced cAMP production and AQP2 targeting in primary cultured IMCD cells, suggesting that ANG II-induced activation of PKC, at least partly, played a role in this process.

**dDAVP or ANG II Has a Stimulatory Effect on AQP2 Targeting to the Plasma Membrane**

Regulation of the water permeability of the apical plasma membrane in the collecting duct principal cells is critical for regulation of renal water reabsorption and body water balance. In the collecting duct, AQP2 is expressed in the apical plasma membrane and subapical vesicles in the collecting duct principal cells and is the chief target for regulation of the osmotic water permeability in response to vasopressin by short-term regulated translocation of intracellular AQP2-bearing vesicles to the apical plasma membrane (37) and by long-term regulation of the AQP2 expression (31). This process allows production of concentrated urine and is essential for water balance regulation.

We demonstrated that acute dDAVP treatment enhanced AQP2 targeting to the plasma membrane of primary cultured IMCD cells. This finding is consistent with a number of previous in vitro and in vivo studies (6, 7, 22, 26, 37, 38, 44, 51, 54). It is well known that increased AQP2 labeling density of the apical plasma membrane in response to vasopressin correlates closely with the increased osmotic water permeability of the isolated perfused IMCD (37). Moreover, vasopressin or dDAVP treatment of vasopressin-deficient Brattleboro rats was associated with a marked increase in AQP2 labeling intensity in the apical plasma membrane of the collecting duct principal cells and an increase in urinary concentration (7, 54). Interestingly, we demonstrated that short-term treatment with ANG II was also associated with increased AQP2 targeting to the plasma membrane in IMCD cells, which was inhibited by...
cotreatment with the ANG II AT\textsubscript{1} receptor blocker candesartan. This suggests that short-term treatment with ANG II could, at least partly, play a role in the activation of signal transduction pathways leading to AQP2 recruitment to the plasma membrane and that this was mediated by ANG II AT\textsubscript{1} receptor activation.

The signal transduction pathways for AQP2 targeting to the plasma membrane have been investigated in a number of previous studies (25, 38). cAMP levels in the collecting duct principal cells are increased in response to vasopressin binding to the V\textsubscript{2} receptor (2, 48, 49), and, subsequently, cAMP activates PKA, which phosphorylates various proteins, including AQP2 (7, 12, 39, 48, 49). Consistent with this, we demonstrated that cAMP levels increased in response to dDAVP in a dose-dependent manner in primary cultured IMCD cells. cAMP levels were also significantly increased by ANG II, and, importantly, dDAVP + ANG II had an additive effect on cAMP production, phosphorylated AQP2 expression, and AQP2 targeting in primary cultured IMCD cells.

We demonstrated enhanced AQP2 targeting to the plasma membrane and increased cAMP levels in response to ANG II with no significant changes in phosphorylated AQP2 expression (Fig. 6). Since intracellular cAMP accumulation in IMCD cells is known to activate PKA, which subsequently increases AQP2 phosphorylation and AQP2 trafficking, an increase in phosphorylated AQP2 expression would be expected. However, at the time point used no significant changes were observed, but it cannot be excluded that AQP2 phosphorylation may have been increased at some time during the stimulation. Moreover, it remains possible that only a minor fraction of AQP2 is subject to phosphorylation and at a level where fractional changes escape detection.

![Image](image_url)
dDAVP + ANG II Potentiates cAMP Production, Phosphorylated AQP2 Expression, and AQP2 Targeting

The actions of the peptide hormones vasopressin and ANG II are mediated by intracellular secondary messengers. Vasopressin induces an increase in intracellular cAMP levels, whereas ANG II induces a rise in [Ca$^{2+}$]i by inositol 1,4,5-triphosphate (4) and PKC activation by diacylglycerol (20). Our findings revealed that ANG II had an additive effect on the dDAVP-induced cAMP production, phosphorylated AQP2 expression, and AQP2 targeting. This suggests cross talk between the intracellular signaling pathways of these two peptide hormones, consistent with our previous findings that ANG II AT1 receptor blockade decreased dDAVP-induced water reabsorption and AQP2 levels in rat kidney (28).

How does short-term treatment with ANG II stimulate cAMP accumulation, AQP2 phosphorylation, and AQP2 targeting in primary cultured IMCD cells? It is known that increases in [Ca$^{2+}$]i and in cAMP levels are required for AQP2 targeting and the accompanying increase in osmotic water permeability (6, 55). The role of Ca$^{2+}$ in the cross talk between ANG II and vasopressin was investigated in a previous study (24) using Chinese hamster ovary cells transfected with cDNA of the AT1a receptor and the V2 receptor. This study demonstrated that chelation of intracellular Ca$^{2+}$ lowered cAMP accumulation by vasopressin and markedly decreased potentiation of vasopressin-induced cAMP accumulation by ANG II (22). On the other hand, the same study also demonstrated that increased [Ca$^{2+}$]i induced by thapsigargin comparable to that induced by ANG II did not potentiate the vasopressin-dependent cAMP accumulation, indicating that the increase in [Ca$^{2+}$]i does not likely play a major role in the cross talk. Consistent with this, Lorenz et al. (29) demonstrated that AQP2 shuttling is evoked neither by a vasopressin-dependent increase of [Ca$^{2+}$]i nor by a vasopressin-independent increase of [Ca$^{2+}$]i in primary cultured IMCD cells from rat kidney, although clamping of [Ca$^{2+}$]i below resting levels inhibits AQP2 exocytosis. However, there is a discrepancy between the results from primary cultured IMCD cells (29) and isolated perfused IMCD tubules (6) with regard to the role of [Ca$^{2+}$]i in vasopressin-induced AQP2 trafficking. Further studies are required to clarify this discrepancy, which may be related to altered expression levels of vasopressin receptors and/or AQP2 or other elements in the AQP2 trafficking system.

Another transduction pathway of ANG II is PKC. In this study, we demonstrated that cAMP levels were significantly increased in response to short-term ANG II treatment, whereas the levels were not increased in response to ANG II treatment in the presence of the PKC inhibitor staurosporine. Moreover, immunocytochemistry demonstrated that AQP2 targeting to the plasma membrane was stimulated by ANG II alone, whereas it was attenuated in response to ANG II + staurosporine. These findings suggest that activation of PKC could, at least in part, play a role in stimulation of adenylate cyclase and AQP2 targeting in primary cultured IMCD cells. It has been shown that PKC activation can inhibit or enhance cAMP accumulation in different cell types (3, 34, 43, 46). The inhibition is frequently caused by phosphorylation and inactivation of receptors linked to adenylate cyclase (34, 46). In contrast, several studies also demonstrated an enhancement of cAMP accumulation by PKC (1, 43, 52). For example, in a system using Swiss 3T3 cells, which provided a useful model to elucidate the early signals and molecular events capable of initiating a mitogenic response, Rozengurt et al. (43) demonstrated that PKC activation by biologically active phorbol esters markedly enhanced cAMP accumulation induced by forskolin and that this was prevented by downregulation of PKC. The modulatory effect of ANG II on adenylate cyclase could involve phosphorylation of different components of the system, including Gs and/or Gi protein, and indeed, several studies suggested that PKC activation of Gi protein may play a role in the stimulation of adenylate cyclase and increase of cAMP levels (40, 43). Another possibility is that the stimula-

![Fig. 8. Immunofluorescent microscopy of AQP2 in primary cultured IMCD cells (protocol 3). A: enhanced AQP2 targeting to the plasma membrane in ANG II-treated cells. B: decreased AQP2 targeting to the plasma membrane in ANG II + PKC inhibitor (staurosporine)-treated cells.](image)

![Fig. 9. cAMP in primary cultured IMCD cells (protocol 3). cAMP was unchanged in response to vehicle or vehicle + PKC inhibitor staurosporine (stau). ANG II increased cAMP, but effect of ANG II + staurosporine was similar to that of vehicle. *P < 0.05 vs. vehicle.](image)
tory effect of PKC on cAMP accumulation could be mediated by interaction with the G1 protein (42), and future studies are warranted to elucidate these possibilities in IMCD cells.

ANG II-mediated intracellular signaling is not restricted to [Ca2+] and PKC: ANG II also activates ERK, JNK, and STAT. In particular, ERK, p38 kinase, and phosphatidylinositol 3’-kinase (PI3-kinase) were shown to regulate AQP2 (5). Importantly, Bustamante et al. (5) demonstrated that vasopressin-induced AQP2 expression was potentiated by insulin in mpkCCD(c14) cells and that the insulin-induced stimulation of AQP2 expression was decreased by inhibition of ERK, p38 kinase, and PI3-kinase activities, whereas inhibition of PKC activity had no effect. Further studies are warranted to examine whether ANG II plays a role in the long-term regulation of AQP2 by increasing AQP2 gene transcription by activation of MAP kinase and PI3-kinase, as demonstrated by insulin (5).

In summary, we demonstrated that short-term ANG II treatment of primary cultured IMCD cells could play a role in the regulation of AQP2 by increasing AQP2 gene transcription by activation of MAP kinase and PI3-kinase, as demonstrated by insulin (5).

REFERENCES


