Mechanisms of Dopamine D$_1$ and Angiotensin Type 2 Receptor Interaction in Natriuresis

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Abstract—Renal dopamine D$_1$–like receptors (D$_1$Rs) and angiotensin type 2 receptors (AT$_2$Rs) are important natriuretic receptors counterbalancing angiotensin type 1 receptor–mediated tubular sodium reabsorption. Here we explore the mechanisms of D$_1$R and AT$_2$R interactions in natriuresis. In uninephrectomized, sodium-loaded Sprague-Dawley rats, direct renal interstitial infusion of the highly selective D$_1$R agonist fenoldopam induced a natriuretic response that was abolished by the AT$_2$R-specific antagonist PD-123319 or by microtubule polymerization inhibitor nocodazole but not by actin polymerization inhibitor cytochalasin D. By confocal microscopy and immunoelectron microscopy, fenoldopam translocated AT$_2$Rs from intracellular sites to the apical plasma membranes of renal proximal tubule cells, and this translocation was abolished by nocodazole. Because D$_1$R activation induces natriuresis via an adenylyl cyclase/cAMP signaling pathway, we explored whether this pathway is responsible for AT$_2$R recruitment and AT$_2$R-mediated natriuresis. Renal interstitial coinfusion of the adenylyl cyclase activator forskolin and 3-isobutyl-1-methylxanthine induced natriuresis that was abolished either by PD-123319 or nocodazole but was unaffected by specific the D$_1$R antagonist SCH-23390. Coadministration of forskolin and 3-isobutyl-1-methylxanthine also translocated AT$_2$Rs to the apical plasma membranes of renal proximal tubule cells; this translocation was abolished by nocodazole but was unaffected by SCH-23390. The results demonstrate that D$_1$R-induced natriuresis requires AT$_2$R recruitment to the apical plasma membranes of renal proximal tubule cells in a microtubule-dependent manner involving an adenylyl cyclase/cAMP signaling pathway. These studies provide novel insights regarding the mechanisms whereby renal D$_1$Rs and AT$_2$Rs act in concert to promote sodium excretion in vivo. (Hypertension. 2012;59[part 2]:437-445.) ● Online Data Supplement

Key Words: dopamine ■ D$_1$ receptors ■ angiotensin ■ AT$_2$ receptors ■ sodium excretion ■ natriuresis

The recycling of membrane proteins is a dynamic process whereby the distribution among different intracellular compartments and the plasma membrane is determined by the rates of exocytosis and endocytosis of the respective membrane proteins. Various stimuli (including agonists and osmotic stress) enhance exocytosis and/or slow endocytosis, leading to a redistribution of membrane proteins to the cell surface, a process termed “translocation.” Translocation often involves an intact cytoskeleton, and the major building blocks of the cytoskeleton are microtubules and actin microfilaments.

Dopamine (DA) receptors belong to 2 receptor subfamilies, D$_1$-like (D$_1$ and D$_3$) and D$_2$-like (D$_2$, D$_3$, and D$_4$). D$_1$-like receptors (D$_1$Rs) are expressed on both apical and basolateral membranes of renal proximal tubule cells (RPTCs). Activation of D$_1$Rs by DA accounts for $\approx$50% of basal sodium ($\text{Na}^+$) excretion in vivo, and intrarenal DA deficiency leads to hypertension and reduced longevity. D$_1$Rs couple to adenylyl cyclase and cAMP generation, as well as phospholipase C and protein kinase C signaling. On agonist stimulation, D$_1$Rs are recruited along microtubules from the interior of RPTCs toward the plasma membrane via cAMP-dependent and not protein kinase C–dependent pathways.

Similar to DA, the angiotensin peptides of the kidney renin-angiotensin system also contribute to the regulation of $\text{Na}^+$ homeostasis through actions at different receptors, including angiotensin type 1 (AT$_1$Rs) and angiotensin type 2 receptors (AT$_2$Rs). In an effort to define the relationship between the renal dopaminergic system and the renin-angiotensin system in $\text{Na}^+$ excretion in normal rodents, previous studies from our laboratory have demonstrated that renal interstitial (RI) D$_1$R activation with fenoldopam (FEN), a highly selective D$_1$R agonist, induces natriuresis that is abolished by intrarenal coinfusion of the specific AT$_2$R antagonist PD-123319 (PD). Furthermore, FEN-induced natriuresis was accompanied by an increase in apical plasma membrane (AM) but not total RPTC membrane AT$_2$R expression, as quantified by Western blot analysis. Because the mechanisms that underlie the trafficking of proteins to and from the cell surface are important determinants of the

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hormonal responsiveness of tissues, the present study examines the roles of the cytoskeleton and cAMP in the redistribution of the natriuretic receptors. We report here that AT$_2$R-mediated natriuresis, either in response to renal D$_1$R stimulation with FEN or direct downstream activation of adenylyl cyclase with forskolin (FSK), results in microtubule-dependent AT$_2$R translocation from the cytosol to the AMs of RPTCs in Na$^+$ loaded Sprague-Dawley rats. Taken together, these findings indicate that cAMP plays an important role in microtubule-dependent trafficking of RPTC AT$_2$Rs, which is necessary for the natriuretic response to D$_1$R activation.

Methods

Animal Preparation

All of the protocols were approved by the animal care and use committee at the University of Virginia and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experiments were conducted on 12-week-old female Sprague-Dawley rats (Harlan) that were housed in a vivarium under controlled conditions (temperature: 21±1°C; humidity: 60±10%; and light: 8:00 AM to 8:00 PM). For 1 week before and during the experiments, the rats were maintained on a standard high Na$^+$ rat chow containing 4% Na$^+$. On day 6, the rats were placed in metabolic cages, and 24-hour urine samples were collected to measure the urine Na$^+$ excretion rate ($U_{\text{Na}}V$). Representative $U_{\text{Na}}V$ was 6.34 mmol/min (normal: 0.69 mmol/min). On day 7, the rats were anesthetized for uninephrectomy, 1-mm coronal sections were cut and placed in 6-well plates with 2 mL of prewarmed medium per well. The sections were equilibrated for 30 minutes in a CO$_2$ incubator at 37°C with gentle rocking before treatment with V, FEN, FEN+NOC, or NOC alone for 30 minutes. Sections were fixed for 2 hours in 4% paraformaldehyde made in Tris-buffered saline at room temperature, rinsed 3 times in Tris-buffered saline, immersed in 100 mmol/L of Tris-HCl, and then rinsed 3 times again in Tris-buffered saline before storage in 30% sucrose in Tris-buffered saline overnight at 4°C. The sections were imbedded in OCT compound (Tissue-Tek), and 8-μm frozen sections were prepared, stained, and analyzed by confocal microscopy, as published previously. Please see the online Data Supplement for details.

Renal Cortical Interstitial Infusion

The RI route of administration was used in these studies to eliminate systemic hemodynamic factors that may play a role in the natriuretic response. The RI catheters were placed as published previously. For 1-hour RI infusion of vehicle (V), followed by 3 hours of RI infusion of one of the following at a rate of 2.5 μL/min: FEN (1, 3, and 5 μg/kg per minute [each dose for 1 hour]; Sigma): 5-nocodazole (NOC, a microtubule polymerization inhibitor; 3 μg/kg per minute; Sigma), NOC alone, FEN+PD (10 μg/kg per minute; Sigma), FEN+cytochalasin D (an inhibitor of actin microfilament polymerization; 0.333 μg/kg per minute; Sigma), cytochalasin D alone, FSK (a direct activator of adenylyl cyclase; 1.4 μg/kg per minute; Sigma): 3-isobutyl-1-methylxanthine (IBMX, a selective phosphodiesterase inhibitor that permits accumulation of cAMP; 1.4 μg/kg per minute; Sigma): nocodazole, IBMX alone, FSK+IBMX+PD, SCH-23390 (SCH, a potent, highly selective D$_1$R antagonist; 10 μg/kg per minute; Sigma): FSK+IBMX, or SCH alone. Control rats received RI V infusion for the entire 4-hour study. When >1 substance was infused, each was infused via a separate microinfusion catheter. Vetbond tissue adhesive (3M Animal Care Products) was added to secure the catheter(s) and prevented interstitial pressure loss in the kidney.

Confocal Microscopy and Quantification of Immunofluorescence Signals

Confocal microscopy recordings were performed using an Olympus IX81 Spinning Disk Confocal Microscopy with excitation at 490 and 467 nm and detection at 510 and 660 nm. Images were captured using identical capture parameters for each section with a ×60 1.2 NA UIS2 water immersion objective and the Sync Measure 3D plug-in written by Joachim Walter, as published previously. The average AT$_2$R fluorescence intensity 0 to 4 μm from the tip of the apical plasma membrane of RPTCs was designated as AM AT$_2$R fluorescence intensity.

Immunoelectron Microscopy

After application of primary and secondary antibodies as described above, the slides were immersed in a solution containing 2.5% glutaraldehyde (EM grade, Electron Microscopy Sciences, Inc [EMS]) in 0.1 mol/L of Dulbecco PBS without calcium and magnesium chloride, held overnight at 4°C, and delivered to the Advanced Microscopy Facility for further processing for transmission electron microscopy. All of the subsequent processing was carried out at 24°C unless otherwise noted. Slides with attached sections were washed in distilled water (4×5 minutes), postfixed for 1 hour in 1% osmium-tetroxide, dehydrated through a graded ethanol series followed by transition into 100% acetone, and infiltrated with epoxy resin (EPON 812, EMS). Although the resin on the slides was

In Vivo Kidney Perfusion and Fixation Procedure

Uninephrectomized Sprague-Dawley rats on a 4% Na$^+$ intake for 1 week received either RI infusion of V, FEN (1 μg/kg per minute), FEN+NOC, or NOC alone for 3 cumulative 1-hour experimental periods. At the end of the in vivo protocol, the rat heart left ventricular cavity was cannulated, and the animal was perfused for fixation before kidney cortex specimen isolation and staining for analysis by quantitative confocal microscopy, as published previously. Please see the online Data Supplement for details.

In Vitro Kidney Section Preparation and Incubation

Kidneys were harvested from animals under anesthesia and immersed in ice-cold RPMI 1640. Using a McIlwaine Tissue Chopper, 1-mm coronal sections were cut and placed in 6-well plates with 2 mL of prewarmed medium per well. The sections were equilibrated for 30 minutes in a CO$_2$ incubator at 37°C with gentle rocking before treatment with V, FEN, FEN+NOC, or NOC alone for 30 minutes. Sections were fixed for 2 hours in 4% paraformaldehyde made in Tris-buffered saline at room temperature, rinsed 3 times in Tris-buffered saline, immersed in 100 mmol/L of Tris-HCl, and then rinsed 3 times again in Tris-buffered saline before storage in 30% sucrose in Tris-buffered saline overnight at 4°C. The sections were imbedded in OCT compound (Tissue-Tek), and 8-μm frozen sections were prepared, stained, and analyzed by confocal microscopy, as published previously. Please see the online Data Supplement for details.

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still liquid, an embedding capsule (BEEM, EMS) filled with epoxide resin was inverted directly over each section and the entire unit (slide with section and capsule) polymerized for 48 hours at 60°C. To separate capsules with underlying embedded tissue sections from the slides, each slide was immersed in boiling water, followed by immersion in liquid nitrogen, and returned to boiling water a second time. Ultrathin sections (70–80 nm) were prepared with a Diatome diamond knife (Diatome, EMS) on a Leica Ultracut UCT ultramicrotome, collected on 200 mesh copper grids (EMS), and contrast stained using a double-lead procedure (Daddow) as follows: 5 minutes in lead citrate; 15 minutes in uranyl acetate (3.0% in 50.0% acetone); and a final 5 minutes in lead citrate. Thin sections were examined in a JEOL 1230 transmission electron microscope (Japan Electron Optics Limited, Tokyo, Japan), and digital images of proximal kidney tubules were acquired with an SIA 12-C slow-scan 16.8 megapixel camera (Scientific Instruments and Applications). Immunogold labeling of AM AT2Rs was quantified from 6 different electron micrographs of RPTC brush borders (each micrograph brush border area: 0.17 $\mu m^2$) for each experimental condition. Immunogold labeling of basolateral membrane AT2Rs was quantified from 5 electron micrographs of RPTC basolateral membrane foldings (totaling an area of 0.25 $\mu m^2$) for each experimental condition.

Membrane Preparations and Western Blot Analysis
After in vitro kidney section preparation, RPTC AMs were isolated as published previously with slight modifications.11,14 Please see the online Data Supplement for details. AMs were incubated with rabbit AT2R polyclonal antibody (1:100 dilution, H-143 Santa Cruz) standardized to villin monoclonal antibody (1:2500 dilution, ImmunoTech), which is enriched in RPTCs.11 Membranes were subsequently incubated with infrared secondary antibodies (antimouse IRDye 680 nm and antirabbit IRDye 800 nm, each at 1:15 000, Licor Biosciences). Immunoreactivity and quantitative assessment of band densities were performed using the Odyssey Infrared Imaging System (Licor Biosciences). Results are reported as a ratio of AT2R:villin expression.

Statistical Analysis
Data are presented as mean±1 SE. ANOVA with a repeated-measures term was used to analyze for variation between the groups. A 2-tailed Student $t$ test was used to compare individual means between groups. A $P<0.05$ was considered statistically significant.

Results

Effects of AT2 Antagonism and Microtubule Polymerization Inhibition on D1R-Induced Natriuresis in Rats on High Na+ Intake
Figure 1A demonstrates that cumulative RI FEN infusion results in increased $U_{NaV}$ across the duration of the experiment and that coinfusion of PD or NOC abolishes this response to control values. FEN increased $U_{NaV}$ from a baseline of 0.23±0.03 to 0.58±0.06 $\mu mol/min$ ($P<0.001$) at 1 $\mu g/kg$ per minute, 0.70±0.07 $\mu mol/min$ ($P<0.0001$) at 3 $\mu g/kg$ per minute, and 0.69±0.07 $\mu mol/min$ ($P<0.0001$) at 5 $\mu g/kg$ per minute. There were no significant changes in $U_{NaV}$ in the V-infused kidneys. As shown in Figure 1B, compared with control kidney sections in which only V was infused, RI FEN, FEN+PD, FEN+NOC, or NOC alone infusions did not alter MAP from baseline values. Identical experiments were performed in which the actin microfilament polymerization inhibitor cytochalasin D failed to affect FEN-induced natriuresis (please see Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org).
Confocal Microscopy Analysis of Renal Proximal Tubule AT$_2$R Redistribution in Response to In Vivo Infusions and In Vitro Incubations

Figure 2A through 2D demonstrate high power (×600) confocal micrographs of the rat renal cortex labeled with phalloidin, a marker for filamentous actin that is enriched in RPTC AM (red), antibodies to the AT$_2$R (green), and Hoechst nuclear stain (blue). As indicated by the colocalization of AT$_2$R and phalloidin (yellow), FEN treatment markedly increased AT$_2$R localization in the apical plasma membrane (AM) of renal proximal tubule cells (RPTCs). The addition of nocodazole (NOC), an inhibitor of microtubule polymerization, to FEN (C and H) abolished AM AT$_2$R recruitment both in vivo and in vitro, whereas NOC treatment alone (D and I) failed to show any difference in AT$_2$R localization compared with vehicle (V) treatment. The bar at the bottom of D and I represents 10 μm. E and J represent the quantification of RPTC AM AT$_2$R fluorescent intensity both in vivo and in vitro, respectively. Compared with V-treated kidneys, (-○-), FEN-treated kidneys (-●-) demonstrated greater RPTC AM AT$_2$R fluorescence intensity both in vivo and in vitro (1765±113 versus 1111±19, P<0.00001, and 1013±20 versus 630±19, P=0.00001, respectively). Both in vivo and in vitro, FEN+NOC treated-kidneys (-•-) abolished the increase in AM AT$_2$R fluorescent intensity, and NOC treatment alone (---) failed to show any significant difference in AT$_2$R localization compared with control conditions (-○-). For in vivo quantifications, each data point represents mean±1 SE of 22 independent measurements of RPTCs. For in vitro quantifications, each data point represents mean±1 SE of 16 independent measurements of RPTCs.

Figure 2. Confocal micrographs (×600) of renal cortical thin sections (8 μm) from both in vivo and in vitro experiments. Vehicle (V)- (A) and fenoldopam (FEN)-treated (B) kidneys and V- (F) and FEN-treated (G) kidney sections stained with Texas-red labeled phalloidin (red), antibody to the angiotensin type 2 receptor (AT$_2$R; green), and Hoechst nuclear stain (blue). As indicated by the colocalization of AT$_2$R and phalloidin (yellow), FEN treatment markedly increased AT$_2$R localization in the apical plasma membrane (AM) of renal proximal tubule cells (RPTCs). The addition of nocodazole (NOC), an inhibitor of microtubule polymerization, to FEN (C and H) abolished AM AT$_2$R recruitment both in vivo and in vitro, whereas NOC treatment alone (D and I) failed to show any difference in AT$_2$R localization compared with vehicle (V) treatment. The bar at the bottom of D and I represents 10 μm. E and J represent the quantification of RPTC AM AT$_2$R fluorescent intensity both in vivo and in vitro, respectively. Compared with V-treated kidneys, (-○-), FEN-treated kidneys (-●-) demonstrated greater RPTC AM AT$_2$R fluorescence intensity both in vivo and in vitro (1765±113 versus 1111±19, P<0.00001, and 1013±20 versus 630±19, P=0.00001, respectively). Both in vivo and in vitro, FEN+NOC treated-kidneys (-•-) abolished the increase in AM AT$_2$R fluorescent intensity, and NOC treatment alone (---) failed to show any significant difference in AT$_2$R localization compared with control conditions (-○-). For in vivo quantifications, each data point represents mean±1 SE of 22 independent measurements of RPTCs. For in vitro quantifications, each data point represents mean±1 SE of 16 independent measurements of RPTCs.
conditions. In vitro FEN-treated RPTCs also demonstrated higher AM AT R fluorescence intensity compared with control conditions (1013 ± 20 versus 630 ± 19; \( P < 0.00001 \); Figure 2J).

Immunoelectron Microscopy Analysis of Renal Proximal Tubule AT R Redistribution After 3-Hour In Vivo Infusions

Figure 3A provides a low-power micrograph of an RPTC. High powered electron photomicrographs (×20,000) of the apical brush border of RPTCs of rat kidneys after the renal interstitial (RI) infusion of vehicle (V; B), fenoldopam (FEN; C), FEN + nocodazole (NOC; D), or NOC alone (F). Black dots represent immunogold (10-nm particles) labeling of brush border angiotensin type 2 receptors (AT R) after each experimental infusion. The bar at the bottom of F represents 0.2 μm. D depicts the quantification of immunogold-labeled AT R densities of RPTC brush borders from 6 different electron micrographs (each micrograph brush border area: 0.17 μm²) for each experimental condition. The electron micrographs confirm increased brush border AT R density after RI infusion of FEN. Data represent the mean ± 1 SE. * \( P < 0.05 \) and ** \( P < 0.01 \) from FEN-infused kidneys.

Role of RI cAMP in AT R-Mediated Natriuresis

After in vivo FSK+IBMX infusion, RI cAMP levels measured via microdialysis increased significantly from a baseline value of 22.1 ± 3.9 to 38.1 ± 5.1 pmol/min (\( P < 0.05 \)) during period 1, 48.6 ± 7.7 pmol/min (\( P < 0.01 \)) during period 2, and 34.8 ± 5.9 pmol/min during period 3 (Figure 4). RI FSK infusion was not sufficient to increase RI cAMP levels significantly. After RI infusion of V (N = 19), FSK alone (N = 12), or IBMX alone (N = 7), \( U_{Na}V \) failed to increase significantly from baseline (Figure 5A). However, after the addition of IBMX to FSK (N = 17), \( U_{Na}V \) increased from 0.21 ± 0.02 to 0.41 ± 0.06 μmol/min in period 1 (\( P < 0.01 \)) to 0.46 ± 0.06 μmol/min in period 2 (\( P < 0.001 \)), and 0.35 ± 0.04 μmol/min in period 3 (\( P < 0.05 \)). The addition of PD (N = 8) or NOC (N = 8) to FSK+IBMX abolished the natriuretic responses (Figure 5A). MAP responses remained unchanged in response to any of the RI infusions (Figure 5B).

Effects of Renal D R Blockade on FSK+IBMX-Induced Natriuresis

As demonstrated in Figure 6A, coinfusion of SCH, an inhibitor of D R Rs, failed to affect FSK+IBMX–induced natriuresis in vivo. In the presence of D R antagonism, FSK+IBMX (N = 12) significantly increased \( U_{Na}V \) from a
baseline value of 0.24±0.02 to 0.37±0.05 μmol/min (P<0.05) during period 1, 0.42±0.05 μmol/min (P<0.05) during period 2, and 0.30±0.03 μmol/min during period 3. RI infusion of SCH alone (N=8) had no effect on UNaV or MAP responses (Figure 6B).

**Western Blot Analysis of Apical Membrane AT2R Expression**

Compared to V-incubated kidneys (N=6), FSK+IBMX (N=6) significantly increased AM AT2R expression (0.0065±0.0004 and 0.0039±0.0003 relative fluorescence units, respectively; P<0.001; Figure 7). The addition of SCH (N=6) did not affect the increase in AM AT2R expression induced by FSK+IBMX treatment. However, none of the individual agents (FSK, IBMX, or SCH alone) altered AM AT2R expression compared with V.

**Discussion**

These studies demonstrate that direct RI D1R activation with FEN, as well as downstream activation of cAMP (in the presence of D1R blockade), induces microtubule-dependent translocation of AT2Rs to the AM of RPTCs and AT2R-mediated natriuresis. Given that renal D1Rs signal through cAMP, and RI accumulation of cAMP is important in the regulation of AT2R-mediated natriuresis, these studies provide novel insight into a mechanism whereby renal D1Rs and AT2Rs act in concert to promote Na\(^+\) excretion in vivo.

It is well known that there is a complex interplay between the intrarenal dopaminergic and renin-angiotensin systems in the control of Na\(^+\) excretion and blood pressure. The natriuretic effect of D1Rs is enhanced during AT1R blockade, and DA, through its actions at D1Rs, decreases AT1R expression and angiotensin II binding sites in RPTCs. Because renal AT2Rs mediate the natriuretic effects of AT1R blockade and specific AT2R antagonism with PD abolishes FEN-induced natriuresis, there exists a clear need to investigate the precise mechanisms by which renal AT2Rs mediate D1R-induced natriuresis. The present studies, therefore, examined the key components of D1R-induced natriuresis.

**Figure 4.** Renal interstitial (RI) cAMP levels in response to the RI infusion of vehicle (V; □; N=10), forskolin (FSK; ■; N=6), and FSK+3-isobutyl-1-methylxanthine (IBMX; □; N=9). Results are reported as picomoles per minute. Data represent the mean±1 SE. *P<0.05 and **P<0.01 from own control.

**Figure 5. A.** Urine sodium excretion (UNaV) in response to renal interstitial (RI) infusion of vehicle (V; □; N=12), forskolin (FSK; ■; N=12), 3-isobutyl-1-methylxanthine (IBMX; □; N=7), FSK+IBMX (□; N=17), FSK+IBMX+PD-123319 (PD; □; N=6), and FSK+IBMX+nocodazole (NOC; □; N=8). Results are reported as micromoles per minute. B. Mean arterial pressure (MAP) in response to conditions in A. Results are reported as millimeters of mercury. Data represent mean±1 SE. *P<0.05, **P<0.01, and ***P<0.001 from own control.
(microtubule-dependent trafficking and cAMP signaling) and their effects on AT$_2$R-mediated Na$^+$ excretion.

First, a dose-response relationship for FEN-induced natriuresis was determined and shown to peak during the 3-$\mu$g/kg-per-minute infusion period. Previous studies carried out under identical experimental conditions using a constant dose of FEN for the entire duration of the study (1 $\mu$g/kg per minute for 3 hours) did not show a peak effect on natriure-

![Graph A](image1)

**Figure 6.** A, Urine sodium excretion ($U_{NaV}$) in response to renal interstitial (RI) infusion of vehicle (V; ■, N=12), SCH-23390 (SCH; ▣, N=8), and SCH+forskolin (FSK)+3-isobutyl-1-methylxanthine (IBMX; ▮, N=12). Results are reported as micromoles per minute. B, Mean arterial pressure (MAP) in response to conditions in A. Results are reported as millimeters of mercury. Data represent mean±1 SE. *P<0.05 from own control.

![Graph B](image2)

![Graph C](image3)

**Figure 7.** B, Western blot analysis of renal proximal tubule cell (RPTC) apical membrane (AM) angiotensin type 2 receptor (AT$_2$R) protein expression in response to vehicle (V), forskolin (FSK), 3-isobutyl-1-methylxanthine (IBMX), FSK+IBMX, SCH-23390 (SCH), SCH+FSK+IBMX, nocodazole (NOC), and FSK+IBMX+NOC treatments in vitro (N=6 for each condition). Data are normalized to villin protein expression and represent mean±1 SE. **P<0.01 and ***P<0.001 vs V treatment.
Thus, intrarenal D₁R activation demonstrates a dose-specific and not time-dependent maximization of Na⁺ excretion. MAP responses were not significantly affected by 3 hours of cumulative FEN infusion, indicating that the observed natriuresis was not a result of systemic hemodynamic factors. Abolition of FEN-induced natriuresis by AT₂R blocker PD occurred as early as the first experimental period with continuation throughout the duration of the protocol, indicating early and sustained AT₂R dependence of D₁R-induced natriuresis. Importantly, the high Na⁺ diet under which these experiments were conducted was demonstrated previously not to reduce RI or tissue levels of angiotensin II/III, which would be available to activate translocated AT₂Rs. AT₂R activation has been shown to induce natriuresis, likely via its downstream NO and cGMP signaling pathways. These observations prompted interest in the acute and nongenomic mechanisms of D₁R-induced AT₂R responses.

One of the results of D₁R activation in salt-loaded animals involved an increase in AM RPTC AT₂R localization. Neither total nor basolateral RPTC AT₂R expression is changed in response to FEN infusion, suggesting that apically distributed AT₂Rs participated in the natriuretic response. Previous experiments have established that D₁Rs translocate to the cell surface in response to D₁R activation in cultured kidney cells, kidney section preparations, and isolated proximal tubules and that this response requires an intact microtubule network. The present studies extend these findings to the natriuretic mechanism of renal AT₂Rs in vivo. Using NOC, which disrupts the microtubule network but preserves the actin microfilaments of RPTCs, we found complete abolition of FEN-induced natriuresis and AT₂R translocation. Although NOC may affect the renal transport of other molecules/receptors involved in D₁R-induced natriuresis in vitro, NOC infusion alone failed to alter basal Na⁺ excretion or AT₂R localization compared with V infusion in vivo. Thus, microtubules are not only necessary for D₁R recruitment but also for AT₂R recruitment in response to FEN, suggesting a common pathway for the natriuretic function of these receptors.

Next, because D₁Rs signal through cAMP/protein kinase A to mediate natriuresis, we examined the role of RI cAMP generation in AT₂R-mediated natriuresis. Although RI FSK infusion alone was insufficient to induce a significant rise in RI cAMP, the addition of IBMX to FSK caused a significant and sustained increase in RI cAMP during the first 2 experimental periods. It is likely that the selective inhibition of cAMP degradation with IBMX was necessary for significant cAMP accumulation because of rapid degradation of the second messenger in vivo. Interestingly, the pattern of increase in UNaV induced by FSK + IBMX paralleled the rise and fall of RI cAMP levels. FSK + IBMX-induced natriuresis was clearly dependent on renal AT₂Rs, because the effect was abolished by PD, but microtubulin-dependent trafficking was also an important component, because NOC also inhibited the effect.

Whether direct agonist stimulation of renal D₁Rs is necessary for AT₂R-mediated natriuresis is a question that was addressed using SCH, a highly specific D₁R antagonist, in conjunction with FSK and IBMX. In the present study, intrarenal D₁R blockade with SCH did not reduce basal Na⁺ excretion acutely, consistent with our previous studies. During SCH administration, RI infusion of FSK + IBMX induced natriuresis that required AT₂R activation, emphasizing the importance of D₁R-induced cAMP generation over direct agonist-dependent activation of D₁Rs. Thus, one mechanism by which AT₂Rs and D₁Rs interact in high Na⁺ conditions to mediate natriuresis is related to D₁R-cAMP signaling, which, in turn, provides the stimulus necessary for AT₂R translocation and natriuresis. Because the effect was independent of specific D₁R-induced activation of adenyl cyclase, stimulation of other receptors that signal through cAMP/protein kinase A pathways may be advantageous in promoting AT₂R-mediated natriuresis in vivo. Indeed, administration of parathyroid hormone, through its cAMP/protein kinase A–dependent but not phospholipase C/protein kinase C–dependent signaling, has been shown to redistribute Na⁺ transporters such as Na⁺−hydrogen exchanger-3 and inhibit Na⁺−K⁺−ATPase activity in a direction favoring natriuresis and diuresis. Renal AT₂Rs are known to inhibit Na⁺−K⁺−ATPase activity, and increased RI cAMP may regulate this process.

In summary, we have shown that renal AT₂Rs are required for D₁R-mediated natriuresis and that both AT₂R cellular trafficking and natriuretic activities are regulated by downstream cAMP signaling pathways. We also have demonstrated that renal AT₂Rs are translocated to the AMs of RPTCs by a microtubule-dependent pathway that is independent of D₁R activation. These results demonstrate an interaction between renal D₁Rs and AT₂Rs that counterbalances Na⁺ reabsorption mediated by AT₂Rs.

**Perspectives**

These studies demonstrate that AT₂R-mediated natriuresis, either in response to renal D₁R stimulation with FEN or direct downstream activation of adenyl cyclase with FSK, involves microtubule-dependent AT₂R translocation to the AMs of RPTCs in Na⁺-loaded rats. Taken together, these findings indicate that cAMP is a key mediator of the interaction between renal D₁Rs and AT₂Rs to induce natriuresis during high-salt states. Furthermore, targeting the common trafficking pathways of these receptors would affect the natriuretic capacity of 2 of the most powerful systems governing Na⁺ homeostasis in the body. Such endeavors hold significant clinical implications in cardiovascular medicine and hypertension where the focus has predominantly been on AT₁R blockade and inhibition of the renin-angiotensin system.

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**Disclosures**

None.
References

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