# Enhanced Natriuresis and Diuresis in Wistar Rats Caused by the Costimulation of Renal Dopamine $D_3$ and Angiotensin II Type 2 Receptors

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**BACKGROUND**

The kidney, via its regulation of sodium excretion, which is modulated by humoral factors, including the dopamine and renin–angiotensin systems, keeps the blood pressure in the normal range. We have reported a negative interaction between dopamine $D_3$ and $AT_1$ receptors ($D_3R$ and $AT_1R$) in renal proximal tubule (RPT) cells. Here, we studied the interaction between $D_3R$ and $AT_2R$ *in vitro* and *in vivo*.

**METHODS AND RESULTS**

Stimulation of either the $D_3R$ or $AT_2R$, by the intrarenal arterial infusion of PD128907, a $D_3R$ agonist, or CGP42112A, an $AT_2R$ agonist, induced natriuresis and diuresis that were enhanced by the simultaneous infusion of PD128907 and CGP42112A in Wistar rats. The $D_3/AT_2$ receptor interaction was confirmed in *in vitro*, i.e., stimulation of either the $D_3R$ or $AT_2R$ inhibited $Na^+$-$K^+$-ATPase activity that was enhanced by the costimulation of these receptors. $D_3R$ and $AT_2R$ colocalized and coimmunoprecipitated in kidney and RPT cells (RPTCs). Stimulation of one receptor increased the localization of the other receptor at the plasma cell membrane. ERK1/2-MAPK is involved in the signaling pathway of $D_3R$ and $AT_2R$ interaction because costimulation of $D_3R$ and $AT_2R$ significantly increased ERK1/2-MAPK expression in RPTCs; inhibition of ERK1/2-MAPK abolished the inhibition of $Na^+$-$K^+$-ATPase activity that was enhanced by $D_3R$ and $AT_2R$ costimulation.

**CONCLUSIONS**

Our current study indicates that $D_3R$, in combination with $AT_2R$, enhances natriuresis and diuresis, via ERK1/2-MAPK pathway, that may be involved in the regulation of blood pressure.

*Keywords:* angiotensin II type 2 receptor; blood pressure; dopamine $D_3$ receptor; hypertension; kidney; kidney tubules; proximal.

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Hypertension, with its complications, is currently a big problem imperiling human health. The mechanisms of hypertension are complex but the kidney plays an important role in blood pressure control by regulating sodium excretion. The proximal tubule (PT) is the major site of salt and water reabsorption in the mammalian nephron, reabsorbing >65% of filtered sodium and water.[1,2] Renal PT (RPT) function is under hormonal control, with angiotensin II stimulating sodium transport, in part, via activation of apical $Na^+$-$H^+$-exchanger-3 (NHE3) and basolateral $Na^+$-$K^+$-ATPase and dopamine inhibiting them.[3]

Dopamine exerts its action via 2 families of dopamine receptors: $D_1$-like receptors ($D_1R$ and $D_5R$) stimulate adenylyl cyclase activity and $D_2$-like receptors ($D_2R$, $D_3R$, and $D_4R$) inhibit adenylyl cyclase.[4] Stimulation of dopamine receptors, especially the $D_1R$ and $D_3R$, induces natriuresis and diuresis.[5–7]

The renin–angiotensin system is a major regulator of renal sodium transport and blood pressure. Angiotensin II is the primary peptide that mediates the effects of the renin–angiotensin system by binding to 2 receptors, $AT_1R$ and $AT_2R$, which have opposing effects. The renal expression of $AT_1R$ is greater than $AT_2R$ which accounts for ~5% of total angiotensin II receptor binding in the RPT. The major effect of angiotensin II, through $AT_1R$, increases sodium transport and aldosterone secretion.[2,3,8,9] Under normal circumstances, the $AT_1R$ masks the renal effects of the $AT_2R$, which can decrease renal sodium transport by inhibiting $Na^+$-$K^+$-ATPase and NHE3 activities in RPTs.[2,10–12]

The renin–angiotensin system and dopaminergic systems interact in the brain[13] and kidney.[3,14–18] For example, $AT_1R$ and $D_1R/D_5R$ negatively interact to regulate renal sodium transport and blood pressure.[14–16] By contrast, $AT_2R$ can mediate the natriuresis induced by $D_1R$.[17] In renal proximal tubule cells (RPTCs) from Wistar-Kyoto rats, $D_3R$ negatively regulates $AT_1R$ expression but this effect is impaired

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in spontaneously hypertensive rats.[18] $D_3R$, as with $AT_2R$, is also expressed in RPT[19] and inhibits sodium transport. Therefore, we tested the hypothesis that $D_3R$ and $AT_2R$ interact to regulate renal sodium transport *in vivo* and *in vitro*.

## MATERIALS AND METHODS

### Animals

The experiments, approved by the Experimental Animals Committee of Daping Hospital, were conducted in 280–300 g Wistar rats. The rats, initially anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg) and maintained by the intravenous infusion of pentobarbital (0.8 mg/100 g/hour), were placed on a heated blanket to maintain rectal temperature ~37 °C and tracheotomized (PE-240). The external jugular and femoral veins were catheterized (PE-50) for fluid administration while the left carotid artery was catheterized (PE-240) for the monitoring of blood pressure. The ureters, exposed via a laparotomy, were catheterized (PE-10) for urine collection. The right suprarenal artery, which originates from the right renal artery, was catheterized (PE-10, heat stretched to 180 μm) for intrarenal vehicle/drug administration (40 μl/hour). Fluid losses during surgery (~60 minutes) were replenished with 5% albumin in normal saline at 1% body weight over 30 minutes. After a 120-minute equilibration period, urine was collected from each ureter for 40 minutes for a total of 5 collection periods. Urinary sodium concentration was measured with an electrolyte analyzer (HC988; Histrong Medical, Shenzhen, China), using ion-selective electrode method.

### Cell culture

Immortalized RPTCs from Wistar-Kyoto rats[15,18,20,21] (passage 20–30) were maintained in Dulbecco's Modified Eagle's medium/F12 medium supplemented with 5% fetal bovine serum, insulin, transferrin, selenium (5 μg/ml each), and epidermal growth factor (10 ng/ml) (GIBCO, MD), at 37 °C in humidified 5% $CO_2$ and 95% air. The cells were serum-starved for 2 hours, treated with vehicle, PD128907 (Tocris, Bristol, UK), CGP42112A, U99194A, PD123319, and PD98059 (Sigma Aldrich, MO), alone or in combination. The antagonist was added 10 minutes before the addition of agonist in the studies involving antagonists.

### Immunoblotting

Immunoblotting was performed as reported.[18,21] The cells were lysed in buffer containing 50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and protease inhibitors. After measuring the protein concentration with bicinchoninic acid kit (Pierce, IL), the protein samples in Laemmli buffer were subjected to 9% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and probed with rabbit anti-phospho-ERK1/2 antibody or anti-ERK1/2 antibody (Cell Signaling Technology, MA), visualized with infrared IRDye antibodies (LI-COR, NE), and scanned by the Odyssey infrared imaging system. The band densities were quantified using the NIH Image J software.

### Coimmunoprecipitation

RPTCs were lysed and renal cortices were homogenized in ice-cold lysis buffer and centrifuged (1,000*g* for 10 minutes) to remove cellular debris. After measuring the protein concentration, 500 μg of cell or tissue lysates were mixed with 2 μg of rabbit anti-$AT_2R$ antibody (Santa Cruz Biotechnology, CA), rocking overnight at 4 °C. Normal rabbit IgG (Santa Cruz Biotechnology) was the negative control and rabbit anti-$D_3R$ antibody (Alpha Diagnostic International) was the positive control. Protein G-agarose beads (Santa Cruz Biotechnology) (30 μl/2 hours) were mixed with the lysates at room temperature. The immune complexes were eluted with 30 μl of 2× Laemmli buffer, boiled, subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotted with rabbit anti-$D_3R$ antibody.[21]

### $Na^+$-$K^+$-ATPase activity assay

$Na^+$-$K^+$-ATPase activity was measured as the rate of inorganic phosphate release in the presence or absence of ouabain.[12,22] The cells was washed twice with chilled phosphate-free buffer (mM: NaCl 3.36, $NaHCO_3$ 0.54, KCl 0.4 and $MgCl_2$ 0.12) and centrifuged at 3,000*g* for 10 minutes. The cell pellets were lysed in buffer (mM: $NaHCO_3$ 1, $CaCl_2$ 2 and $MgCl_2$ 5) and centrifuged at 3,000*g* for 2 minutes. The supernatant was mixed in sodium iodide (1 M) and centrifuged at 48,000*g* for 25 minutes to obtain membrane pellets. The pellets were washed and suspended in Tris–HCl (mM: Tris 10 and EDTA 1, pH 7.4). The protein concentrations were quantified with bicinchoninic acid assay. Hundred microliters of membrane suspension were mixed with 800 μl reaction mixture A (mM: NaCl 70, KCl 5, $MgCl_2$ 5, $Na_4EGTA$ 1, $NaN_3$ 6, imidazole 37.5, Tris–HCl 75; pH 7.4) for measurement of total ATPase activity and reaction mixture B (mM: $MgCl_2$ 5, $Na_4EGTA$ 1, $NaN_3$ 6, imidazole 37.5, Tris–HCl 75; pH 7.4) (Sigma Aldrich) with ouabain (Sigma Aldrich) (1 mM) for measurement of ouabain-insensitive ATPase activity. Reactions were initiated by the addition of ATP (4 mM), incubated at 37 °C/15 minutes, and terminated by the addition of trichloroacetate (50%). The tubes were placed on ice for 2 minutes. One milliliter of coloring reagent (5% $FeSO_4$ in 1% ammonium molybdate in 1N sulfuric acid) was added into the reaction mixtures, mixed, and centrifuged at 3,000*g* for 10 minutes. The amount of inorganic phosphate in the supernatants was quantified spectrophotometrically at 740 nm. A standard curve was constructed using $KH_2PO4$. $Na^+$-$K^+$-ATPase activity, which was the difference between total and ouabain-insensitive ATPase activity, was normalized with protein concentration and activity expressed as nmol phosphate released per mg protein per minute.

### Immunofluorescence and confocal microscopy

For immunofluorescence studies, kidney sections were deparaffinized, rehydrated, and subjected to antigen retrieval using citric acid buffer (10 mM, pH 6.0). RPTCs on coverslips in 24-well plates were fixed with 4% paraformaldehyde. $D_3R$ was immunostained with goat anti-$D_3R$ antibody (Santa

Cruz Biotechnology), followed by Cy3-labeled donkey anti-goat antibody (Beyotime Institute of Biotechnology, Haimen, China); $AT_2R$ was immunostained with rabbit anti-$AT_2R$ antibody, followed by Alexa fluor 488 goat anti-rabbit antibody (Molecular Probes, OR). Secondary antibodies from different species were used to avoid cross-reactivity; incubation of donkey anti-goat antibody was performed prior to the goat anti-rabbit antibody. The images were obtained using laser confocal microscopy.

### Statistical analysis

Data are expressed as mean ± SEM. Significant differences within groups were determined by 1-way repeated measures ANOVA, followed by Holm-Sidak *post hoc* test. Significant differences among groups were determined by 1-way factorial ANOVA, followed by Holm-Sidak test. $P < 0.05$ was accepted as statistically significant.

## RESULTS

### Enhanced natriuretic and diuretic effect of renal $D_3R$ and $AT_2R$ costimulation in Wistar rats

To determine the effect of $D_3R$ on sodium excretion, varying dosages of $D_3R$ agonist, PD128907 (0.5, 1.0, 5.0 μg/kg/minute × 40 minutes), were infused into the right renal artery in Wistar rats. The intrarenal arterial infusion of the vehicle into the right kidney had no effect on urine flow (V) and absolute sodium excretion ($U_{Na}V$) (Figure 1a1,a2). However, the intrarenal arterial infusion of PD128907 increased V and $U_{Na}V$, with significant effects first observed at 1.0 μg/kg/minute (Figure 1a1,a2). The specificity of PD128907 as a $D_3R$ agonist was verified by the coinfusion of a $D_3R$ antagonist, U99194A, at a dose that by itself had no effect on V or $U_{Na}V$. In the presence of U99194A (5.0 μg/kg/minute), the PD128907 (1.0 μg/kg/minute)-induced diuresis and natriuresis were completely blocked (Figure 1b1,b2). The intrarenal arterial infusion of PD128907, U99194A, or their combination did not affect blood pressure (Supplementary Figure S1A,B).

We next investigated the effect of $AT_2R$ on V and $U_{Na}V$. CGP42112A, an $AT_2R$ agonist, infused at 0.5, 1.0, 5.0 μg/kg/minute × 40 minute), induced a diuresis and natriuresis in a dose-dependent manner with significant effects first observed at a dose of 1.0 μg/kg/minute (Figure 1a1,a2). In the presence of PD123319 (5.0 μg/kg/minute), an $AT_2R$ antagonist, the CGP42112A (1.0 μg/kg/minute)-induced diuresis and natriuresis were completely blocked (Figure 1c1,c2). The intrarenal arterial infusion of CGP42112A, PD123319, or the combination of CGP42112A and PD123319 did not affect blood pressure (Supplementary Figure S1A,C).

Consistent with the studies shown in Figure 1a,b, the intrarenal infusion of 0.5 μg/kg/minute of either PD128907 or CGP42112A did not significantly induce diuresis or natriuresis (Figure 1d1,d2; Tables 1 and 2). However, the simultaneous infusion of 0.5 μg/kg/minute of both PD128907 and CGP42112A produced a greater than an additive increase in V and $U_{Na}V$ in periods 2, 3, and recovery. The enhanced increase in V and $U_{Na}V$ was blocked by either $D_3R$ or $AT_2R$ antagonist. The intrarenal arterial infusion of $D_3R$ and $AT_2R$ agonist, antagonist, or their combination did not affect blood pressure (Supplementary Figure S1D).

### Enhanced inhibition of $Na^+$-$K^+$-ATPase activity by $D_3R$ and $AT_2R$ costimulation in RPTCs

Consistent with our previous report,[23] the stimulation of $D_3R$ with PD128907 ($10^{-10}$–$10^{-7}$ M, 15 minutes) inhibited $Na^+$-$K^+$-ATPase activity in a concentration-dependent manner, with a significant effect first observed at $10^{-8}$ M, in immortalized RPTCs (Figure 2a). The inhibitory effect of PD128907 ($10^{-8}$ M) was specific to the $D_3R$ because its effect was blocked by the $D_3R$ antagonist U99194A ($10^{-5}$ M) (Figure 2b). CGP42112A ($10^{-10}$–$10^{-7}$ M, 15 minutes) also inhibited $Na^+$-$K^+$-ATPase activity in a concentration-dependent manner, with a significant effect first observed at $10^{-9}$ M, in immortalized RPTCs (Figure 2a). The inhibitory effect of CGP42112A ($10^{-9}$ M) was specific to the $AT_2R$ because its effect was blocked by the $AT_2R$ antagonist PD123319 ($10^{-6}$ M) (Figure 2c). The $D_3R$ antagonist U99194A and $AT_2R$ antagonist PD123319, by themselves, had no effect on basal $Na^+$-$K^+$-ATPase activity.

The costimulation of $D_3R$, with the lowest concentration of PD128907 ($10^{-8}$ M) that inhibited $Na^+$-$K^+$-ATPase activity and a concentration of CGP42112A ($10^{-10}$ M) that did not affect $Na^+$-$K^+$-ATPase activity, inhibited $Na^+$-$K^+$-ATPase activity to a greater extent than that caused by PD128907 ($10^{-8}$ M), alone, in RPTCs (Figure 2d; Table 3), indicating an enhanced rather than additive effect, which is similar to that observed with the increase in V and $U_{Na}V$ observed *in vivo*. The presence of either their respective antagonist (U99194A, $D_3R$, or PD123319, $AT_2R$) blocked the enhanced inhibition of $Na^+$-$K^+$-ATPase activity by $D_3R$ and $AT_2R$ costimulation (Figure 2d).

### Colocalization of $D_3R$ and $AT_2R$ in the kidney of Wistar rats

$D_3R$ and $AT_2R$ were both expressed in RPTs of Wistar rats, in agreement with previous reports.[11,19] There was colocalization of $D_3R$ and $AT_2R$ in RPTs (Figure 3a). There was also a physical interaction between $D_3R$ and $AT_2R$; renal cortex homogenates immunoprecipitated with anti-$AT_2R$ antibody and immunoblotted with anti-$D_3R$ antibody, revealed a band that corresponded with the $D_3R$ (Figure 3b). The $D_3R$ and $AT_2R$ also colocalized and coimmunoprecipitated in immortalized RPTCs. Stimulation of either $D_3R$ or $AT_2R$ minimally increased the $D_3R$ and $AT_2R$ colocalization. However, the stimulation of RPTCs with the lowest concentration of PD128907 ($10^{-8}$ M) that inhibited $Na^+$-$K^+$-ATPase activity and a concentration of CGP42112A ($10^{-10}$ M) that did not inhibit $Na^+$-$K^+$-ATPase activity increased their colocalization in RPTC membranes and cytoplasm (Figure 3c). Under basal conditions, endogenously expressed $D_3R$ and $AT_2R$ were both membrane-bound and scattered in the cytoplasm, with slight intracellular colocalization. Activation of either $D_3R$ or $AT_2R$ resulted in granular staining at the membrane and cytoplasm but simultaneous stimulation of $D_3R$ and $AT_2R$ led to a strong granular staining of both receptors at the plasma membrane and cytoplasm, subjacent to

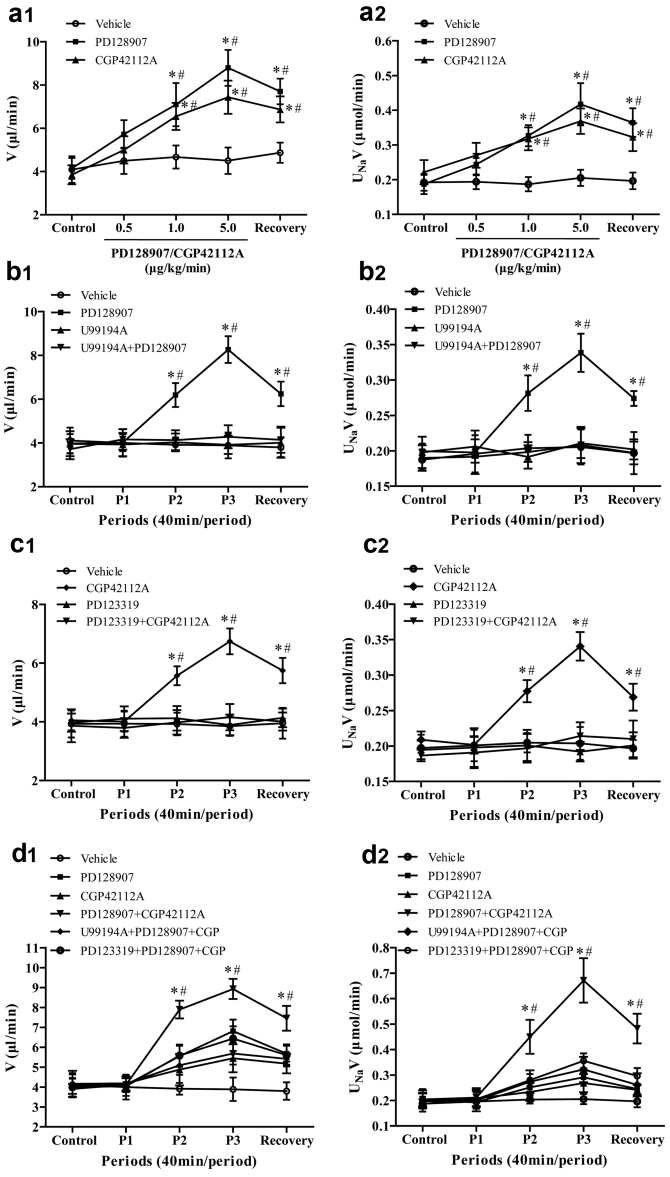


**Figure 1.** Effect of the intrarenal arterial infusion of $D_3R$ and $AT_2R$ agonist and/or antagonist on urine flow and sodium excretion in Wistar rats. There were 4 series of studies. The effects of the $D_3R$ agonist PD128907 and $AT_2R$ agonist CGP42112A were studied in the first series (**a1**: urine flow [V] and **a2**: sodium excretion [$U_{Na}V$]). The effects of the $D_3R$ agonist PD128907 and $D_3R$ antagonist U99194A were studied in the second series (**b1** and **b2**). The effects of the $AT_2R$ agonist CGP42112A and $AT_2R$ antagonist PD123319 were studied in the third series (**c1** and **c2**). The effects of the $D_3R$ agonist PD128907, $D_3R$ antagonist U99194A, $AT_2R$ agonist CGP42112A, $AT_2R$ antagonist PD123319, and their combinations were studied in the fourth series. There were 5 urine collection periods, each period lasting for 40 minutes. During the control period, vehicle (normal saline) was infused followed by vehicle (Vehicle Group) or drug infusion periods and a recovery period in which only vehicle (normal saline) was infused in all groups. Figures a1 and a2 show the effect of $D_3R$ agonist PD128907 or $AT_2R$ agonist CGP42112A on V and $U_{Na}V$. PD128907 or CGP42112A was infused at 0.5, 1.0, and 5.0 μg/kg/minute. Data are expressed as mean ± SEM, $^*P < 0.05$ vs. each Control (repeated measures analysis of variance [ANOVA], Holm-Sidak test). $^{\#}P < 0.05$ vs. Vehicle, $n = 6$ (1-way factorial ANOVA, Holm-Sidak test). Figures b1 and b2 show the effect of $D_3R$ antagonist U99194A on the $D_3R$ agonist PD128907-mediated effect on V and $U_{Na}V$. During drug infusion period 1 (P1), $D_3R$ antagonist U99194A (5 μg/kg/minute) was infused into U99194A and PD128907 + U99194A groups. The drug infusion (P2 and P3) groups consisted of the $D_3R$ agonist PD128907 (1.0 μg/kg/minute), $D_3R$ antagonist U99194A (5 μg/kg/minute), or their combination (PD128907 + U99194A). Data are expressed as mean ± SEM, $^*P < 0.05$ vs. Control (repeated measures ANOVA, Holm-Sidak test). $^{\#}P < 0.05$ vs. other groups, $n = 5$–6 (1-way factorial ANOVA, Holm-Sidak test). Figures c1 and c2 show the effect of $AT_2R$ antagonist PD123319 on the $AT_2R$ agonist CGP42112A-mediated effect on V and $U_{Na}V$. During drug infusion period 1 (P1), $AT_2R$ antagonist PD123319 (5 μg/kg/minute) was infused into PD123319 and CGP42112A + PD123319 groups. The drug infusion (P2 and P3) groups consisted of the $AT_2R$ agonist CGP42112A (1.0 μg/kg/minute), $AT_2R$ antagonist PD123319 (5 μg/kg/minute), or their combination (CGP42112A + PD123319). Data are expressed as mean ± SEM, $^*P < 0.05$ vs. Control (repeated measures ANOVA, Holm-Sidak test). $^{\#}P < 0.05$ vs. other groups, $n = 5$–6 (1-way factorial ANOVA, Holm-Sidak test). Figures **d1** and **d2** show the effect of $D_3R$ agonist (PD128907), $D_3R$ antagonist (U99194A), $AT_2R$ agonist (CGP42112A), $AT_2R$ antagonist (PD123319), or their combinations on V and $U_{Na}V$. During drug infusion period 1 (P1), $D_3R$ antagonist U99194A (5 μg/kg/minute) and $AT_2R$ antagonist PD123319 (5 μg/kg/minute) were infused into PD128907 + CGP42112A + U99194A and PD128907 + CGP42112A + PD123319 group, respectively. The drug infusion (P2 and P3) groups consisted of PD128907 (0.5 μg/kg/minute), CGP42112A (0.5 μg/kg/minute), PD128907 + CGP42112A, PD128907 + CGP42112A + U99194A, and PD128907 + CGP42112A + PD123319. Data are expressed as mean ± SEM, $^*P < 0.05$ vs. Control (repeated measures ANOVA, Holm-Sidak test). $^{\#}P < 0.05$ vs. other groups, $n = 5$–6 (1-way factorial ANOVA, Holm-Sidak test).

**Table 1.** Effect of $D_3R$ agonist or antagonist, $AT_2R$ agonist or antagonist, alone or in combination on urine flow in Wistar rats

| | Control | P1 | P2 | P3 | Recovery |
|---|---|---|---|---|---|
| Vehicle | 4.11 ± 0.60 | 4.0 ± 0.62 | 3.91 ± 0.30 | 3.89 ± 0.59 | 3.80 ± 0.44 |
| PD128907 (PD) | 3.95 ± 0.47 | 4.11 ± 0.36 | 5.55 ± 0.52 | 6.81 ± 0.58[a,c] | 5.68 ± 0.40 |
| CGP42112A (CGP) | 4.16 ± 0.65 | 4.18 ± 0.35 | 4.87 ± 0.79 | 5.45 ± 0.71 | 5.18 ± 0.49 |
| PD128907 + CGP42112A | 3.91 ± 0.25 | 4.11 ± 0.33 | 7.90 ± 0.45[a,b] | 8.93 ± 0.51[a,b] | 7.46 ± 0.62[a,b] |
| U99194A + PD128907 + CGP | 4.06 ± 0.58 | 4.17 ± 0.35 | 5.10 ± 0.48 | 5.69 ± 0.55 | 5.41 ± 0.30 |
| PD123319 + PD128907 + CGP | 3.99 ± 0.46 | 4.05 ± 0.49 | 5.58 ± 0.56 | 6.43 ± 0.62[a,c] | 5.60 ± 0.54 |

Wistar rats were treated as described in Figure 1d.
[a]$P < 0.05$ vs. Control (repeated measures analysis of variance [ANOVA], Holm-Sidak test). [b]$P < 0.05$ vs. others, [c]$P < 0.05$ vs. Vehicle, $n = 5–6$ (1-way factorial ANOVA, Holm-Sidak test).

**Table 2.** Effect of $D_3R$ agonist or antagonist, $AT_2R$ agonist or antagonist, alone or in combination on sodium excretion in Wistar rats

| | Control | P1 | P2 | P3 | Recovery |
|---|---|---|---|---|---|
| Vehicle | 0.187 ± 0.0195 | 0.196 ± 0.0285 | 0.204 ± 0.0158 | 0.205 ± 0.0202 | 0.197 ± 0.0236 |
| PD128907 (PD) | 0.197 ± 0.0109 | 0.193 ± 0.0094 | 0.251 ± 0.0165 | 0.291 ± 0.0199[a,c] | 0.245 ± 0.0207 |
| CGP42112A (CGP) | 0.202 ± 0.0256 | 0.207 ± 0.0293 | 0.233 ± 0.0355 | 0.268 ± 0.0354 | 0.242 ± 0.0198 |
| PD128907 + CGP42112A | 0.204 ± 0.0265 | 0.211 ± 0.0373 | 0.450 ± 0.0667[a,b] | 0.671 ± 0.0871[a,b] | 0.482 ± 0.0585[a,b] |
| U99194A + PD128907 + CGP | 0.205 ± 0.0351 | 0.199 ± 0.0421 | 0.274 ± 0.0450 | 0.322 ± 0.050 | 0.261 ± 0.0455 |
| PD123319 + PD128907 + CGP | 0.201 ± 0.0441 | 0.205 ± 0.0280 | 0.280 ± 0.0240 | 0.356 ± 0.0292[a,c] | 0.296 ± 0.0314 |

Wistar rats were treated as described in Figure 1d.
[a]$P < 0.05$ vs. Control (repeated measures analysis of variance [ANOVA], Holm-Sidak test). [b]$P < 0.05$ vs. others, [c]$P < 0.05$ vs. Vehicle, $n = 5–6$ (1-way factorial ANOVA, Holm-Sidak test).

the plasma membrane, and increased their colocalization. There are some differences in the cellular localization of the $D_3R$ in the current report from our previous report in which $D_3R$ stimulation resulted in its internalization with the endothelin B (ETB) receptor in the same RPTCs.[7] These differences may relate to a shorter duration of incubation and lower concentration of the $D_3R$ agonist (PD128907 $10^{-8}$ M, 15 minute vs. PD128907 $10^{-6}$ M, 30 minute) and different receptors studied that interact with the $D_3R$ ($AT_2R$ vs. ETB receptor), different sources (Alpha Diagnostic International vs. Zymed) and types (polyclonal vs. monoclonal) of the $D_3R$ antibodies. A coimmunoprecipitation study confirmed the interaction. Stimulation of RPTCs with the lowest concentration of PD128907 ($10^{-8}$ M) that inhibited $Na^+$-$K^+$-ATPase increased the coimmunoprecipitation of $D_3R$ and $AT_2R$. The concentration of CGP42112A ($10^{-10}$ M) that did not inhibit $Na^+$-$K^+$-ATPase activity did not increase the coimmunoprecipitation of $D_3R$ and $AT_2R$ but their combination increased their coimmunoprecipitation to a greater extent than that caused by PD128907 ($10^{-8}$ M), alone, in RPTCs (Figure 3d).

### MAPK is involved in the enhanced effect of $D_3R$ and $AT_2R$ costimulation in RPTCs

MAPK and extracellular signal-regulated kinase (ERK) are involved in the signaling pathway of both $D_3R$ and $AT_2R$.[10,24] Therefore, we determined if $D_3R$ and $AT_2R$ affect ERK phosphorylation (p-ERK). Similar to the experiments in Figure 2d, we used the lowest concentration of PD128907 ($10^{-8}$ M) that inhibited $Na^+$-$K^+$-ATPase activity and a concentration of CGP42112A ($10^{-10}$ M) that did not inhibit $Na^+$-$K^+$-ATPase activity. We found that PD128907 ($10^{-8}$ M) increased p-ERK while CGP42112A ($10^{-10}$ M) did not affect p-ERK. The addition of the nonstimulatory effect (p-ERK) of CGP42112A ($10^{-10}$ M) almost doubled the ability of PD128907 ($10^{-8}$ M) to increase p-ERK expression (Figure 4a). The presence of a MAPK inhibitor, PD98059 ($10^{-5}$ M), reduced the enhanced effect of $D_3R$ and $AT_2R$ costimulation on $Na^+$-$K^+$-ATPase activity (Figure 4b). The presence of the MAPK inhibitor PD98059 ($10^{-5}$ M) also decreased the colocalization and coimmunoprecipitation of $D_3R$ and $AT_2R$ induced by $D_3R$ (PD128907, $10^{-8}$ M) and $AT_2R$ (CGP42112A, $10^{-10}$ M) agonists in RPTCs (Figures 4c,d). The enhanced colocalization of the $D_3R$ and $AT_2R$ at the membrane and cytoplasm, subjacent to the membrane with the combination of PD128907 and CGP42112A, was partially abolished by the MAPK inhibitor PD98059.

## DISCUSSION

Interactions among G protein-coupled receptors are important not only in their normal function but also in the pathogenesis of disease.[7,18,25,26] Heteromerization of receptors leads to modulation of signaling and activating properties of individual G protein-coupled receptors. For example,

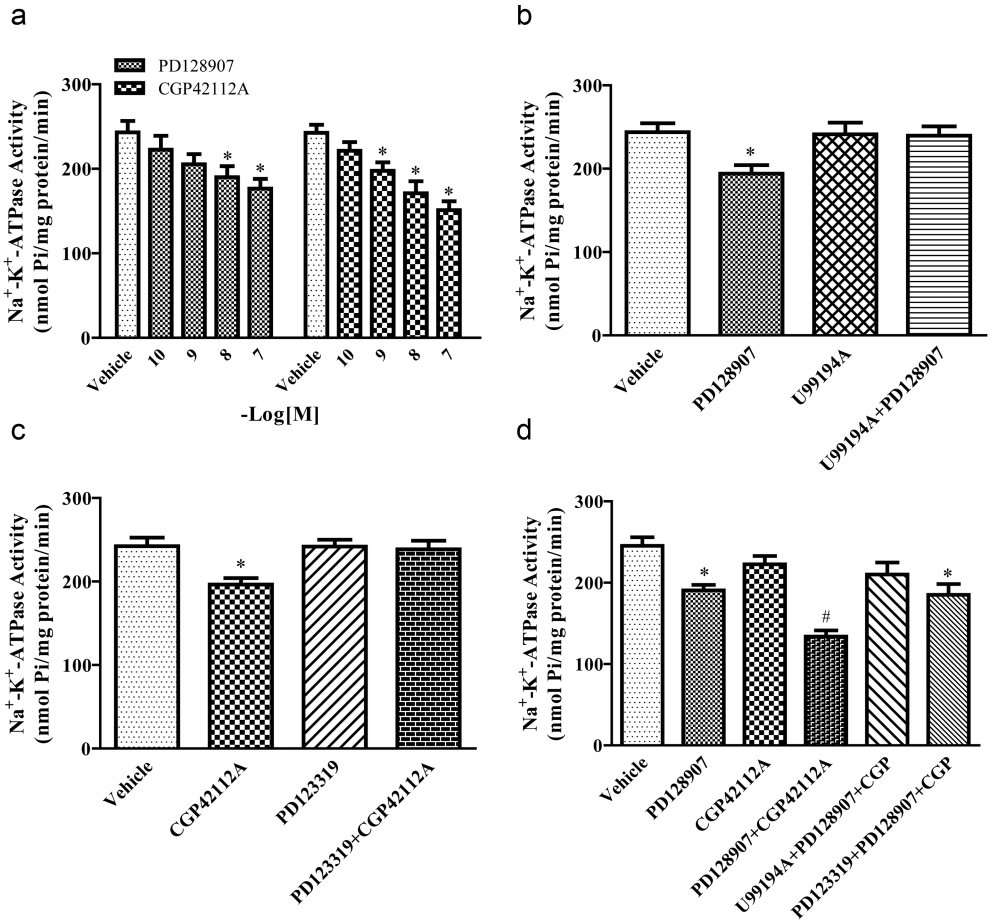


**Figure 2.** Effect of $D_3R$ and $AT_2R$ costimulation on $Na^+$-$K^+$-ATPase activity in renal proximal tubule cells (RPTCs). (**a**) Effect of the $D_3R$ agonist PD128907 or the $AT_2R$ agonist CGP42112A on $Na^+$-$K^+$-ATPase activity. RPTCs were treated with varying concentrations of PD128907 or CGP42112A ($10^{-10}$–$10^{-7}$ M) for 15 minutes. Protein concentration was used to normalize $Na^+$-$K^+$-ATPase activity. Data are expressed as mean ± SEM. *$P$ < 0.05 vs. respective Vehicle, $n$ = 6 (1-way analysis of variance [ANOVA], Holm-Sidak test). (**b**) Effect of the $D_3R$ agonist PD128907, $D_3R$ antagonist U99194A, alone or in combination on $Na^+$-$K^+$-ATPase activity in RPTCs. RPTCs were pretreated with vehicle or U99194A ($10^{-5}$ M) for 10 minutes, then treated with PD128907 ($10^{-8}$ M) for 15 minutes. Protein concentration was used to normalize the results. Data are expressed as mean ± SEM. *$P$ < 0.05 vs. others, $n$ = 6 (1-way ANOVA, Holm-Sidak test). (**c**) Effect of the $AT_2R$ agonist CGP42112A, $AT_2R$ antagonist PD123319, alone or in combination on $Na^+$-$K^+$-ATPase activity in RPTCs. RPTCs were pretreated with vehicle or PD123319 ($10^{-6}$ M) for 10 minutes, then treated with CGP42112A ($10^{-9}$ M) for 15 minutes. Protein concentration was used to normalize the results. Data are expressed as mean ± SEM. *$P$ < 0.05 vs. others, $n$ = 7 (1-way ANOVA, Holm-Sidak test). (**d**) Effect of $D_3R$ and $AT_2R$ agonists and antagonists on $Na^+$-$K^+$-ATPase activity. The effect of the $D_3R$ agonist PD128907 ($10^{-8}$ M), $D_3R$ antagonist U99194A ($10^{-5}$ M), $AT_2R$ agonist CGP42112A ($10^{-10}$ M), $AT_2R$ antagonist PD123319 ($10^{-6}$ M), alone or in combination (PD128907 + CGP42112A; PD128907 + CGP42112A + U99194A; PD128907 + CGP42112A + PD123319) on $Na^+$-$K^+$-ATPase activity. RPTCs were pretreated with vehicle, U99194A, or PD123319 for 10 minutes, then treated with PD128907 and CGP42112A, alone or in combination for 15 minutes. Drugs were mixed immediately before treatment; protein concentration was used to normalize the results. Data are expressed as mean ± SEM. *$P$ < 0.05 vs. Vehicle. #$P$ < 0.05 vs. others, $n$ = 6–8 (1-way ANOVA, Holm-Sidak test).

**Table 3.** Effect of $D_3R$ and $AT_2R$ agonist, antagonist, alone or in combination on $Na^+$-$K^+$-ATPase activity in RPTCs

| Treatment | $Na^+$-$K^+$-ATPase activity |
|---|---|
| Vehicle | 245.27 ± 10.70 |
| PD128907(PD) | 190.60 ± 6.87[a] |
| CGP42112A (CGP) | 222.61 ± 10.26 |
| PD128907 + CGP42112A | 133.87 ± 7.50[b] |
| U99194A + PD128907 + CGP42112A | 210.13 ± 14.81 |
| PD123319 + PD128907 + CGP42112A | 185.22 ± 13.06[a] |

RPTCs were treated as described in Figure 2d.
Abbreviation: RPTC, renal proximal tubule cells.
[a]$P$ < 0.05 vs. Vehicle, [b]$P$ < 0.05 vs. others, $n$ = 6–8 (1-way ANOVA, Holm-Sidak test).

the $D_1R$ and $D_3R$ interact to decrease sodium transport in RPTCs and relax vascular smooth muscle cells.[21,27,28]

There are many reports on the interaction of the dopaminergic and renin–angiotensin system in the regulation of renal sodium excretion and blood pressure.[3] All the dopamine receptor subtypes can negatively interact with $AT_1R$.[14–16,18,29–31] The $AT_2R$ can mediate the natriuresis induced by $D_1$-like receptors.[17] However, an interaction between $D_2$-like receptors and the $AT_2R$ has not been reported. We now report an enhanced effect of $D_3R$ and $AT_2R$ costimulation in decreasing $Na^+$-$K^+$-ATPase activity and increasing sodium excretion. The $D_3R$ and $AT_2R$ probably regulate each other by physical interaction. We show that these receptors colocalize and coimmunoprecipitate in renal cortex homogenates and RPTCs. This physical interaction between $D_3R$

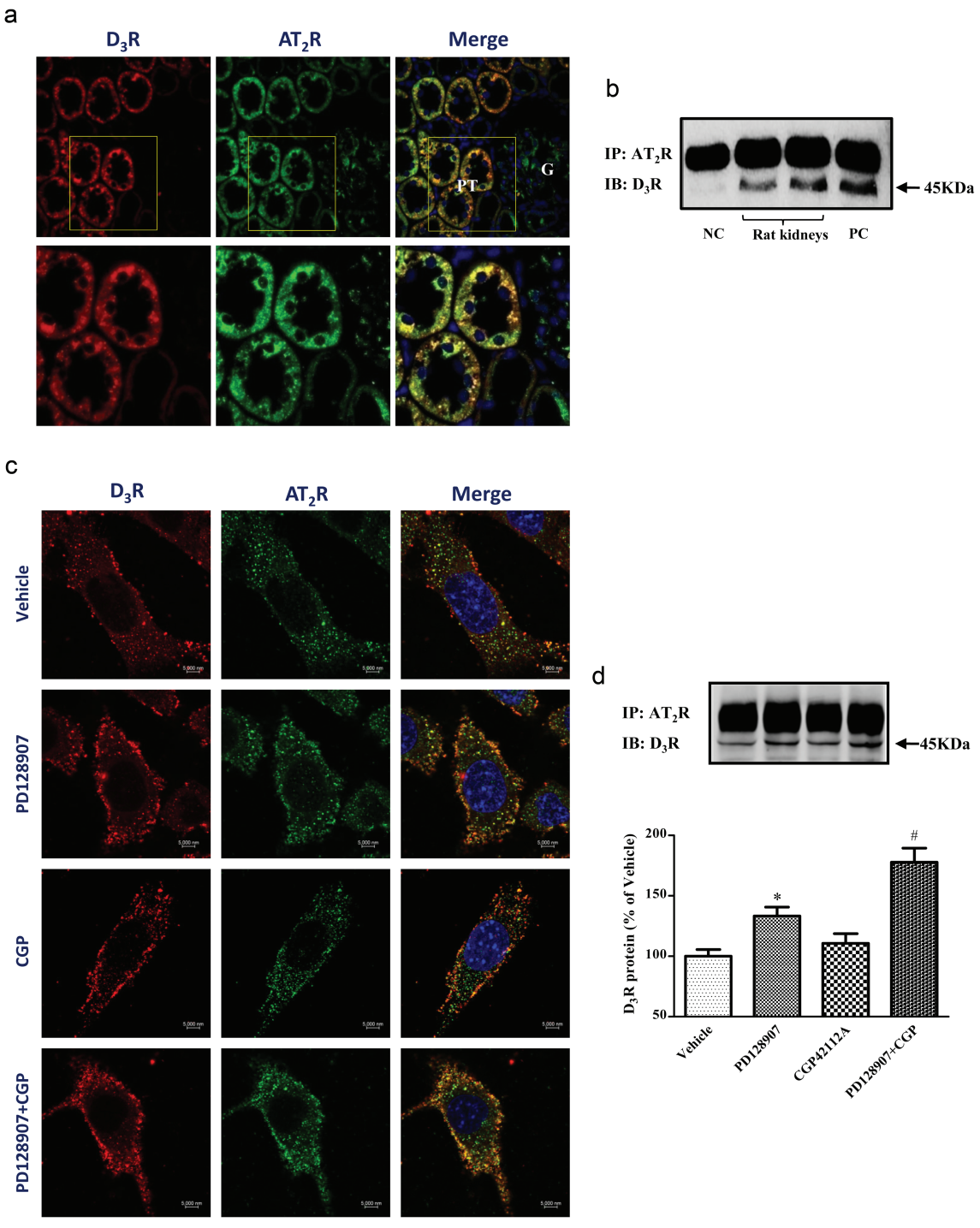


**Figure 3.** Colocalization and coimmunoprecipitation of $D_3R$ and $AT_2R$ in Wistar rat kidney and renal proximal tubule cells (RPTCs). (**a**) Cellular distribution and colocalization of $D_3R$ and $AT_2R$ in rat kidney cortex. Formalin-fixed, paraffin-embedded sections of rat renal cortex were stained with anti-$D_3R$ and anti-$AT_2R$ antibodies. Colocalization was determined by confocal microscopy. $D_3R$ was pseudocolored red and $AT_2R$ was pseudocolored green; the yellow areas show their colocalization in the merged images. The nuclei are stained blue; PT, proximal tubule; G, glomerulus. Images are representative of 3 experiments using different kidney sections. (**b**) $D_3R$ and $AT_2R$ physically interact in the rat kidney. Renal cortex lysates were immunoprecipitated with anti-$AT_2R$ antibody and immunoblotted with anti-$D_3R$ antibody. Normal rabbit IgG served as a negative control, anti-$D_3R$ antibody served as positive control. (**c**) Cellular distribution and colocalization of $D_3R$ and $AT_2R$ in RPTCs. The cells were treated with vehicle, and PD128907 ($10^{-8}$ M) and CGP42112A ($10^{-10}$ M), or their combination for 15 minutes. $D_3R$ was pseudocolored red and $AT_2R$ was pseudocolored green; the yellow areas show their colocalization in the merged images. The nuclei are stained blue. Images are representative of 3 experiments using different cell preparations. (**d**) Coimmunoprecipitation of $D_3R$ and $AT_2R$ in RPTCs. The cells were treated with vehicle, and PD128907 ($10^{-8}$ M) and CGP42112A ($10^{-10}$ M), or their combination for 15 minutes. The cell lysates were immunoprecipitated with anti-$AT_2R$ antibody and immunoblotted with anti-$D_3R$ antibody. Data are expressed as mean ± SEM. $^{*}P < 0.05$ vs. Vehicle, $^{\#}P < 0.05$ vs. others, $n = 4$ (1-way analysis of variance, Holm-Sidak test).

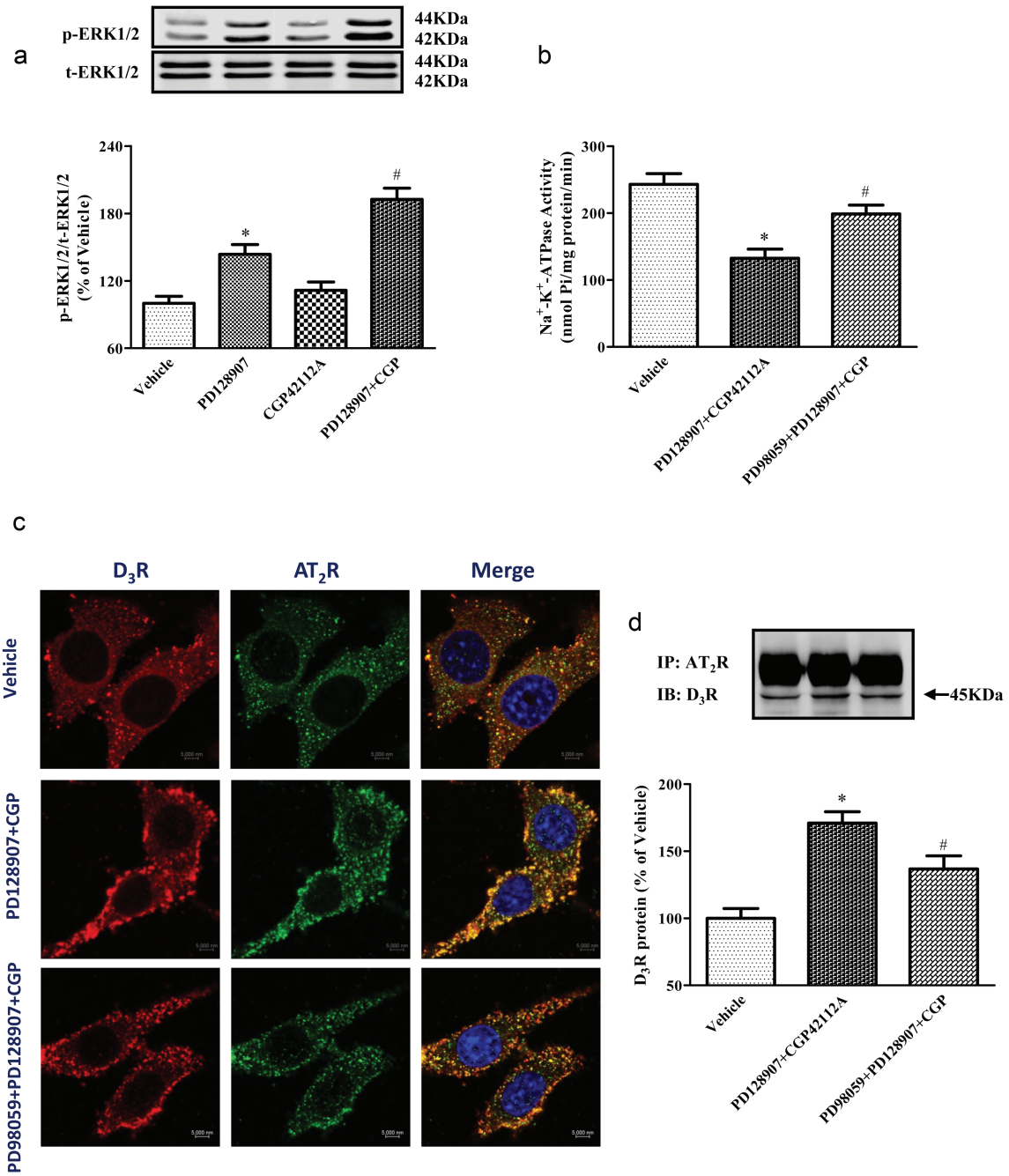


**Figure 4.** Effect of MAPK inhibitor on $D_3R$ and $AT_2R$ agonist-mediated effects on phospho-ERK, $Na^+$-$K^+$-ATPase activity, and $D_3R$ and $AT_2R$ colocalization and coimmunoprecipitation in renal proximal tubule cells (RPTCs). (**a**) Effect of $D_3R$ and $AT_2R$ agonists on phospho-ERK1/2 (p-ERK1/2) protein expression in RPTCs. RPTCs were treated with vehicle, and the $D_3R$ agonist, PD128907 ($10^{-8}$ M), and $AT_2R$ agonist CGP42112A ($10^{-10}$ M), or their combination. The cell lysates were immunoblotted with anti-phospho-ERK antibody and anti-total (t) ERK antibody. P-ERK expression was corrected by t-ERK protein expression. Data are expressed as mean ± SEM. $^*P < 0.05$ vs. Vehicle, $^{\#}P < 0.05$ vs. others, $n = 5$ (1-way analysis of variance [ANOVA], Holm-Sidak test). (**b**) Effect of $D_3R$ and $AT_2R$ agonists on the $Na^+$-$K^+$-ATPase activity of RPTCs in the absence or presence of MAPK inhibitor PD98059. RPTCs were pretreated with vehicle or PD98059 ($10^{-5}$ M) for 10 minutes, then treated with PD128907 ($10^{-8}$ M) in combination with CGP42112A ($10^{-10}$ M) for 15 minutes. Protein concentration was used to normalize the results. Data are expressed as mean ± SEM. $^*P < 0.05$ vs. Vehicle, $^{\#}P < 0.05$ vs. others, $n = 6$ (1-way ANOVA, Holm-Sidak test). (**c**) Effect of $D_3R$ and $AT_2R$ agonists on the cellular distribution and colocalization of $D_3R$ and $AT_2R$ in RPTCs in the absence and presence of MAPK inhibitor PD98059. RPTCs were pretreated with PD98059 ($10^{-5}$ M) for 10 minutes, then treated with PD128907 ($10^{-8}$ M) in combination with CGP42112A (CGP, $10^{-10}$ M) for 15 minutes. Colocalization of $D_3R$ (red) and $AT_2R$ (green) was observed as discrete yellow areas in merged images. Images are representative of 3 experiments using different cell preparations. (**d**) Effect of $D_3R$ and $AT_2R$ agonists on the coimmunoprecipitation of $D_3R$ and $AT_2R$ in RPTCs in the absence and presence of MAPK inhibitor PD98059. RPTCs were pretreated with vehicle or PD98059 ($10^{-5}$ M) for 10 minutes, then treated with PD128907 ($10^{-8}$ M) in combination with CGP42112A (CGP, $10^{-10}$ M) for 15 minutes. Data are expressed as mean ± SEM. $^*P < 0.05$ vs. Vehicle, $^{\#}P < 0.05$ vs. others, $n = 6$ (1-way ANOVA, Holm-Sidak test).

and $AT_2R$ is important in the inhibition of $Na^+$-$K^+$-ATPase activity that is mediated by the MAPK and ERK pathway. As indicated earlier, G protein-coupled receptor signaling and function can be affected by heteromerization.[21,26–28] Based on those reports, we presume that $D_3R$ and $AT_2Rs$ may also heterodimerize and enhance receptor crosstalk and downstream signal transduction, leading to amplification of their effects and inhibition of sodium transport, in this instance.[25]

We used CGP42112A, a peptide $AT_2R$ agonist; a nonpeptide $AT_2R$ agonist, Compound 21,[32] as with CGP42112A, has a high affinity for $AT_2R$.[33] CGP42112A is easily degraded whereas Compound 21 may not.[34] The current experiments were performed in the short term to avoid possible pharmacokinetic problems and used a low concentration ($10^{-10}$ M) of CGP42112A that acts as a full agonist without antagonist effect.[35] The natriuretic effect of $AT_2R$ stimulation may be due to renal tubular rather than a hemodynamic effect.[36,37] We have reported that the intrarenal infusion of $D_3R$ agonist did not affect glomerular filtration rate,[7] suggesting that the natriuretic effect of $D_3R$ stimulation could be also attributed to tubular rather than hemodynamic effect. However, it should be noted that the natriuresis observed in the current study (~0.5 μmol/minute) is not large and could be accounted for by a small increase in glomerular filtration rate (<0.3%). The proximal tubules, which is the major site of sodium reabsorption in the nephron,[1,2] also express the $D_3R$ and $AT_2R$.[8,19] However, nephron segments beyond the proximal tubule could also be involved in the natriuresis due to $D_3R$ and $AT_2R$ interaction.[7,38] We also studied only the short-term effect of the intrarenal arterial infusion of drugs to avoid the confounding influence of alterations in systemic hemodynamic or hormones, such as aldosterone. However, urinary potassium was not monitored in the current study. Our results show that the antagonists of $D_3R$ and $AT_2R$, by themselves, do not affect $Na^+$-$K^+$-ATPase activity or sodium excretion, suggesting absence of constitutive activity, at least in the kidney.[38] Another $D_3R$ antagonist, GR103691, did not affect renal sodium excretion in Wistar-Kyoto rats on high-salt diet,[7] in agreement with our results.

Owing to the low expression of $AT_2R$ in adult kidney,[8,11] renal endogenous $AT_2R$ effect on sodium transport is masked by $AT_1R$.[9,39] However, $AT_2R$ can directly interact with $AT_1R$ in immortalized RPTCs from Wistar-Kyoto rats; $AT_2R$ stimulation inhibited $AT_1R$ expression as early as 8 hours, lasting for at least 30 hours. In these RPTCs treated with $AT_2R$ agonist for 24 hours, the decrease in $AT_1R$ protein was associated with a decrease in angiotensin II-mediated stimulation of $Na^+$-$K^+$-ATPase activity.[22] In these RPTCs, we also reported that $D_3R$ stimulation decreased $AT_1R$ protein as early as 2 hours, lasting for at least 24 hours.[18] In the current study, short-term (40 minutes) $D_3R$ stimulation did not change $AT_1R$ expression. Therefore, the enhancement of sodium and water excretion by the costimulation of the $D_3R$ and $AT_2R$ is probably caused by an increase in their physical interaction rather than a change in the amount of receptor expression. This may be the reason why the functional interaction between the $D_3R$ and $AT_2R$ did not last for a long time. Our results show that MAPK and ERK participate in the enhancement of $D_3R$ and $AT_2R$-induced natriuresis and diuresis. NO/cGMP signaling cascade is also involved in $AT_2R$-mediated natriuresis,[39] however, NO does not participate in $D_3R$ signaling.[40] The role of the heterodimerization of $D_3R$, $AT_2R$, and $AT_1R$[18,22] in their interaction needs to be studied.

In summary, stimulation of $D_3R$ or $AT_2R$ induces diuresis and natriuresis; costimulation of $D_3R$ and $AT_2R$ produces an enhanced effect, which may be mediated by their physical interaction. MAPK and ERK are involved in the signaling pathway of the $D_3R$ and $AT_2R$ receptor-enhanced effect.

## SUPPLEMENTARY MATERIAL

Supplementary materials are available at *American Journal of Hypertension* (http://ajh.oxfordjournals.org).

## ACKNOWLEDGMENTS

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## DISCLOSURE

The authors declare no conflict of interest.

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