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The Dopamine D_1 Receptor and Angiotensin Type-2 Receptor are Required for Inhibition of Sodium Transport Through a Protein Phosphatase 2A Pathway

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Abstract

Activation of the renal dopamine D_1-like receptor (D_1R) or angiotensin II type-2 receptor (AT_2R), individually or both, simultaneously, is necessary in the normal regulation of renal sodium (Na^+) transport and blood pressure. However, little is known regarding the precise mechanism of this interaction. Pharmacologic stimulation, membrane biotinylation, and cell surface immunofluorescence were used to study the effect of the D_1R/AT_2R interaction in human renal proximal tubule cells (RPTCs). D_1R activation of $G\alpha_S$ stimulates adenylyl cyclase and induces apical plasma membrane recruitment of AT_2Rs. We now show for the first time the reciprocal reaction, AT_2R stimulation with angiotensin III leads to the apical plasma membrane recruitment of the D_1R. The cell-permeable second messenger analogs of cAMP (8-Br-cAMP) or cGMP (8-Br-cGMP) induce translocation of both D_1R and AT_2R to the plasma membrane. Inhibition of protein kinase A (PKA) with Rp-cAMPS and protein kinase G (PKG) with Rp-8-CPT-cGMPS blocks D_1R and AT_2R recruitment, respectively, indicating that both PKA and PKG are necessary for D_1R and AT_2R trafficking. Both 8-Br-cAMP and 8-Br-cGMP activate protein phosphatase 2A (PP2A) which is necessary for both plasma membrane recruitment of D_1R and AT_2R and the inhibition of NHE3-dependent Na^+ transport. These studies provide insights into the D_1R/AT_2R transregulation mechanisms that play a crucial role in maintaining Na^+ and ultimately blood pressure homeostasis.

Summary

This study extends previous observations about the D_1R and AT_2R role in regulating Na^+ transport by demonstrating in human RPTCs that there is an absolute co-dependency between D_1R and AT_2R to inhibit Na^+ transport despite their differences in second messenger linkage.

Keywords

Dopamine 1 receptor; Angiotensin 2 receptor; Protein Phosphatase 2A; Renal sodium transport

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INTRODUCTION

The renal dopaminergic and renin-angiotensin systems are major regulators of sodium (Na^+) homeostasis[1–4]. Both dopamine D_1-like receptors (D_1R) and angiotensin II type-2 receptors (AT_2R) are natriuretic receptors, as stimulation by either dopamine or angiotensin III (Ang III), respectively, inhibits renal Na^+ reabsorption, resulting in natriuresis[4,5]. Upon agonist stimulation, D_1Rs are recruited by microtubules from the cytoplasm to the apical cell surface[6,7]. Renal tubular plasma membrane D_1Rs are further stimulated by dopamine synthesized in the renal proximal tubule cell (RPTC) that is released in a paracrine manner, increasing G protein coupling and a subsequent activation of adenylyl cyclase (AC) and protein kinase A (PKA)[6,8]. Renal angiotensin II (Ang II) is converted to Ang III by aminopeptidase A, which stimulates AT_2Rs to decrease Na^+ transport through an increase in soluble guanylyl cyclase (GC) production of cGMP which stimulates protein kinase G (PKG)[9]. (Figure 6).

Previous studies have not determined whether the D_1R or AT_2R acts independently or collectively to inhibit renal Na^+ transport. In rodents, AT_2Rs are recruited to the RPTC apical plasma membrane upon D_1R stimulation and the AT_2R antagonist, PD-123319 (PD), blocks the D_1R-mediated natriuretic response, suggesting that AT_2R is necessary for D_1R activity[10]. In human RPTCs, we and others have reported that D_1R and AT_2R activation increases cAMP and cGMP, respectively, leading to a decrease in Na^+ transport[11–14], but the interdependence of D_1R and AT_2R stimulation on receptor translocation to the apical plasma membrane is unknown. The goal of this study is to investigate further the relationship between the D_1R and AT_2R in human RPTCs. We have reported that the resensitization of the desensitized, phosphorylated D_1R is dependent on its dephosphorylation by protein phosphatase 2A (PP2A)[15–18]. The current study tests the hypothesis that downstream activation of either D_1R or AT_2R converges at PP2A which is necessary for both receptors to be translocated to the apical cell surface to inhibit Na^+ transport.

MATERIAL AND METHODS

The authors declare that the data that support the findings of this study are available from the corresponding author upon reasonable request.

Pharmacological agents

All pharmacological agents were purchased from Sigma-Aldrich. Fenoldopam, (FEN) is a D_1R agonist; it stimulates the two D_1-like receptors, D_1R and D_5R. 7-Methyl-6,7,8,9,14,15-hexahydro-5H-benz[d]indolo[2,3-g]azecine (LE300[19]) is a potent and selective D_1R antagonist[20]. H-Arg-Val-Tyr-Ile-His-Pro-Phe-OH, Angiotensin III (Ang III) is the preferred endogenous AT_2R agonist[21]. 1-[[4-(Dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid ditrifluoroacetate, PD-123319 (PD) is a potent and selective non-peptide antagonist to the AT_2R[22]. 8-Bromoadenosine cyclic 3',5'-monophosphate (8-Br-cAMP) is a cell-permeable cAMP analog. 8-Bromoguanosine cyclic 3',5'-monophosphate (8-Br-cGMP) is a cell-permeable cGMP analog[23]. Rp-adenosine 3′,5′-cyclic monophosphorothioate

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triethylammonium salt (Rp-cAMPS) is a PKA antagonist[24]. 8-(4-chlorophenylthio) guanosine-3,5-cyclic monophosphorothioate, Rp-isomer triethylammonium salt (Rp-8-CPT-cGMPS) is a PKG antagonist[25]. 9,10-Deepithio-9,10-didehydroacanthifolicin, Okadaic Acid (OA) is a PP2A antagonist[14]. Ouabain (OUB) is a Na^+/K^+ATPase alpha inhibitor. 3,3′-(4-Methyl-1,2-phenylene)bis[N-(aminoiminomethyl)-2-methyl-2-propenamide] dihydrochloride, S3226, is a potent and selective NHE3 inhibitor[26]

Human RPTCs

Human kidney tissue and cell culture procedures were followed as reported previously[27,28]. For more details, please see the Online Supplement.

Quantification of Cell Surface Recruitment of D_1R and AT_2R by Flow Cytometry

RPTCs were grown in iPT-G418 media in 100 mm plates for 2 days until 60–80% confluence at 37°C in full humidity with 5% CO_2. Vehicle (control) and agonists were added to cells and inhibitors were added 15 min prior to the addition of receptor agonists. Cells were stained in suspension and stained without permeabilization according to details found in the online supplement. Treated and stained cells were run on a benchtop flow cytometer with a robotic autoloader (Accuri C6Sampler, Accuri Cytometers).

Quantification of Cell Surface Recruitment of D_1R and AT_2R by 96-Well In-Cell Western

Procedures were followed as reported previously[29]. For more details, please see the Online Supplement.

Biotinylation and Western Blot Analysis

Procedures were followed as reported previously[14,28]. For more details, please see the Online Supplement.

PP2A Activity Assay

RPTCs at 80% confluence in 100 mm plates were incubated in serum-free DMEM-F12 media with or without agonists, washed, and lysed in phosphate-free buffer, according to kit instructions (Millipore, cat# 17–313). Briefly, 500 μg of protein were immunoprecipitated using a monoclonal antibody specific for the catalytic subunit of PP2A (clone 1D6). Phosphatase activity was measured by the release of phosphate from a phosphorylated peptide K-R-pT-I-R-R using malachite green by absorbance at 620 nm in a 96-well plate reader (BMG, Pherastar FSX) and compared to a standard curve made by diluting phosphate directly in malachite green.

NHE3-Mediated Na^+ Accumulation Assay

Procedures were followed as reported previously[26]. For more details, please see the Online Supplement.

Specific Protocols

1. Effects of D_1-Like Agonist FEN±D_1R Antagonist LE300 and the Effects of Endogenous AT_2R Agonist Ang III±AT_2R Antagonist PD on D_1R and AT_2R

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Recruitment Measured by Flow Cytometry.—Human RPTCs were treated as follows: (1) VEH (N=12); normal media, (2) FEN (N=6); 1 μmol/L for 30 min, (3) FEN+LE300 (N=6); pretreated with LE300 (1 μmol/L) for 15 min followed by 30 min co-incubation with FEN, (4) LE300 (N=6); 30 min, (5). Ang III (N=6); 10 nmol/L for 30 min, (6) Ang III+PD (N=6); pretreated with PD (1 μmol/L) for 15 min followed by 30 min co-incubation with Ang III, and (7) PD (N=6); 30 min.

2. Dose Response Curves for 8-Br-cAMP and 8-Br-cGMP to Measure Maximal D_1R and AT_2R Recruitment by Flow Cytometry.—Human RPTCs were treated as follows: 8-Br-cAMP and 8-Br-cGMP for 30 min (N=6 per dose): 10 mmol/L, 3 mmol/L, 1 mmol/L, 0.3 mmol/L, 0.1 mmol/L, 0.03 mmol/L, and 0.01 mmol/L.

3. Effects of 8-Br-cAMP and 8-Br-cGMP on D_1R and AT_2R Recruitment Measured by Flow Cytometry and Western Blot Analysis.—Human RPTCs were treated as follows: (1) VEH (N=15); normal media, (2) 8-Br-cAMP (N=15); 1 mmol/L for 30 min, (3) 8-Br-cGMP (N=15); 1 mmol/L for 30 min, and (4) 8-Br-cAMP+8-Br-cGMP (N=15); 30 min. In a separate set of experiments (N=3 for each condition), RPTCs were treated with either 8-Br-cAMP or 8-Br-cGMP to measure both D_1R and AT_2R recruitment by Western blot analysis.

4. Effects of 8-Br-cGMP±PKG Inhibitor Rp-8-CPT-cGMPS and the Effects of 8-Br-cAMP±PKA Inhibitor Rp-cAMPS on D_1R and AT_2R Recruitment Measured by Flow Cytometry.—Human RPTCs were treated as follows: (1) VEH (N=12), normal media, (2) 8-Br-cGMP (N=6); 1 mmol/L for 30 min, (3) 8-Br-cGMP+Rp-8-CPT-cGMPS (N=6); pretreated with Rp-8-CPT-cGMPS (100 μmol/L) for 15 min followed by 30 min co-incubation with 8-Br-cGMP, (4) Rp-8-CPT-cGMPS (N=6); 30 min, (5) 8-Br-cAMP (N=6); 1 mmol/L for 30 min, (6) 8-Br-cAMP+Rp-cAMPS (N=6); pretreated with Rp-cAMPS (100 μmol/L) for 15 min followed by 30 min co-incubation with 8-Br-cAMP, and (7) Rp-cAMPS (N=6); 30 min.

5. Effects of 8-Br-cAMP and 8-Br-cGMP on PP2A Activity.—Studies followed the same protocol stated in Protocol 3 and N=3 for each condition.

6. Effects of PP2A Inhibition with OA on 8-Br-cAMP- and 8-Br-cGMP-Induced D_1R and AT_2R Recruitment Measured by Flow Cytometry.—Human RPTCs were treated as follows: (1) VEH (N=6); normal media, (2) 8-Br-cAMP (N=6); 1 mmol/L for 30 min, (3) 8-Br-cGMP (N=6); 1 mmol/L for 30 min, (4) 8-Br-cAMP+OA (N=6); pretreated with OA (10 nmol/L) for 15 min followed by 30 min co-incubation with 8-Br-cAMP, (5) 8-Br-cGMP+OA (N=6); pretreated with OA for 15 min followed by 30 min co-incubation with 8-Br-cGMP, and (6) OA (N=6); 30 min.

7. Effects of PP2A Inhibition with OA on Na^+ Transport.—Human RPTCs were treated as follows: (1) VEH (N=6); normal media, (2) 8-Br-cAMP (N=6); 1 mmol/L for 30 min, (3) 8-Br-cGMP (N=6); 1 mmol/L for 30 min, (4) 8-Br-cAMP+8-Br-cGMP (N=6); both for 30 min, (5) 8-Br-cAMP+OA (N=6); pretreated with OA (10 nmol/L) for 15 min followed by 30 min co-incubation with 8-Br-cAMP, (6) 8-Br-cGMP+OA (N=6); pretreated with OA

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for 15 min followed by 30 min co-incubation with 8-Br-cGMP, (7) 8-Br-cAMP+8-Br-cGMP +OA (N=6); pretreated with OA for 15 min followed by the co-incubation with 8-Br-cAMP +8-Br-cGMP, and (8) OA (N=6); 30 min.

Statistical Analysis

The data are presented as mean ± SEM. One-way ANOVA, followed by Tukey's test was used to compare 3 groups. A two-tailed Student's t-test was used to compare 2 groups. P<0.05 was considered statistically significant.

RESULTS

Flow Cytometry Analysis of the Effects of Exogenously Added FEN and Ang III on D_1R and AT_2R Recruitment.

Figure 1A. RPTCs stimulated with FEN (1 μmol/L, 30 min) increased the recruitment of the D_1R to the apical plasma membrane (1.56±0.07-fold, FEN vs. VEH, P<0.05, N=6). The addition of the D_1R antagonist LE300 (1 μmol/L, 30 min) + FEN significantly inhibited D_1R membrane recruitment (FEN vs. FEN+LE300, P<0.05, N=6 per group), while LE300, alone, had no effect compared to VEH. Figure 1B. RPTCs stimulated with FEN also increased the recruitment of the AT_2R to the apical plasma membrane (1.53±0.12-fold, FEN vs. VEH, P<0.05, N=6 per group), which was inhibited in the presence of LE300 (FEN vs. FEN +LE300, P<0.05, one-way ANOVA, N=6 per group). LE300 alone had no effect on AT_2R recruitment compared to VEH. Figure 1C. RPTCs stimulated with Ang III (10 nmol/L, 30 min) increased recruitment of the D_1R to the apical plasma membrane (1.39±0.06-fold, Ang III vs. VEH, P<0.05, N=6 per group). The addition of the AT_2R antagonist PD (1 μmol/L, 30 min) + Ang III significantly inhibited D_1R membrane recruitment (Ang III vs. AngIII+PD, P<0.05, N=6 per group), while PD alone had no effect compared to VEH. Figure 1D. RPTCs stimulated with Ang III increased the apical plasma membrane recruitment of the AT_2R (1.49±0.10-fold, Ang III vs. VEH, P<0.05, N=6 per group). The addition of the AT_2R antagonist PD+Ang III significantly inhibited AT_2R membrane recruitment (Ang III vs. AngIII+PD, P<0.05, N=6 per group), while PD alone, had no effect compared to VEH. This is the first time AT_2R stimulation with Ang III is shown to induce D_1R apical plasma membrane recruitment.

Dose-Response Analysis of 8-Br-cAMP and 8-Br-cGMP

Dopamine binding to the D_1R leads to AC activation and accumulation of cAMP[30]. Similarly, Ang III binding to the AT_2R leads to GC activation and accumulation of cGMP[31]. Using cell permeable analogs of cAMP and cGMP, 8-Br-cAMP and 8-Br-cGMP, respectively, allows us to study downstream signaling leading to D_1R and AT_2R membrane trafficking. Concentration-response curves were measured for both 8-Br-cAMP and 8-Br-cGMP stimulation of D_1R and AT_2R apical plasma membrane recruitment, using a variant of an In-Cell Western blotting technique, where the directly labeled antibodies are added to cells without cell permeabilization. Semi-log dilutions from 10 mmol/L to 10 nmol/L for both 8-Br-cAMP and 8-Br-cGMP were added to RPTCs for 30 min and both D_1R and AT_2R plasma membrane recruitment were analyzed. A concentration-response curve for 8-Br-cAMP and 8-Br-cGMP showed maximal recruitment of the D_1R and AT_2R at 1 mmol/L for

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both 8-Br-cAMP Figure S1A and S1B and 8-Br-cGMP Figure S1C and S1D. For the D_1R, the EC_{50} of 8-Br-cAMP was 296.1 μmol/L (Log EC_{50} was −3.529) and the EC_{50} of 8-Br-cGMP was 144.3 μmol/L (Log EC_{50} was −3.841). For the AT_2R the EC_{50} of 8-Br-cAMP was 206.4 μmol/L (Log EC_{50} was −3.685) and the EC_{50} of 8-Br-cGMP was 165.6 μmol/L (Log EC_{50} was −3.781).

Flow Cytometry Analysis of 8-Br-cAMP, 8-Br-cGMP, or 8-Br-cAMP+8-Br-cGMP Stimulation on D_1R and AT_2R Recruitment

The Emax of 1 mmol/L, determined from Figure 2 for both 8-Br-cAMP and 8-Br-cGMP, measured by In-Cell Western experiments, was confirmed using flow cytometry (Figure 2A and 2B). 8-Br-cAMP, 8-Br-cGMP, or 8-Br-cAMP+8-Br-cGMP significantly increased the plasma membrane recruitment of D_1R (1.50±0.08-, 1.51±0.08-, and 1.56±0.0-fold, respectively, $P<0.05$, N=15 per group). 8-Br-cAMP, 8-Br-cGMP, or 8-Br-cAMP+8-Br-cGMP also significantly increased the plasma membrane recruitment of AT_2R (1.58±0.07-, 1.61±0.06-, and 1.58±0.08-fold respectively, $P<0.05$, N=15 per group). Interestingly, the simultaneous addition of both 8-Br-cAMP and 8-Br-cGMP did not cause an additional increase in apical plasma membrane recruitment, relative to either agonist alone.

Biotinylation and Western Blot Analysis of 8-Br-cAMP and 8-Br-cGMP Stimulation on D_1R and AT_2R Recruitment

Biotinylation and Western blot analysis were performed to verify the results generated from flow cytometry and In-Cell Western experiments. We measured D_1R (Figure 2C) and AT_2R (Figure 2D) recruitment when the cells were stimulated with 8-Br-cAMP and 8-Br-cGMP (both at 1 mmol/L) for 30 min. RPTCs stimulated with 8-Br-cAMP and 8-Br-cGMP induced an increase in apical plasma membrane-localized D_1R expression by 2.71±0.21-fold ($P<0.05$) and 1.98±0.31-fold ($P<0.05$), N=3 per group, respectively. 8-Br-cAMP and 8-Br-cGMP also induced an increase in apical plasma membrane-localized AT_2R expression by 2.11±0.42-fold ($P<0.05$) and 1.64±0.10-fold ($P<0.05$), N=3 per group respectively. These results are consistent with the flow cytometry results, indicating that the flow cytometry technique is a reliable method for measuring receptor recruitment.

Flow Cytometry Analysis of the Effects of PKG and PKA Inhibitors on Cell Surface D_1R and AT_2R Recruitment.

In order to determine, more precisely, the signaling downstream of cAMP and cGMP, we next examined the effect of PKA and PKG inhibition on D_1R and AT_2R recruitment in RPTCs. PKA and PKG are inhibited by Rp-cAMPS and Rp-8-CPT-cGMPS, respectively for 15 min prior to 8-Br-cAMP and 8-Br-cGMP stimulation. Exogenous 8-Br-cGMP (1 mmol/L, 30 min) increased the apical plasma membrane recruitment of D_1R (1.56±0.05-fold vs. VEH, *$P<0.05$, N=6), which was inhibited in the presence of the PKG inhibitor Rp-8-CPT-cGMPs, (100 μmol/L, #$P<0.05$, 8-Br-cGMP+Rp-c8 vs. 8-Br-cGMP, N=6). Rp-8-CPT-cGMPS, alone, had no effect (Figure 3A). Exogenous 8-Br-cAMP (1 mmol/L, 30 min) recruited AT_2Rs to the cell surface (1.58±0.04-fold vs. VEH, *$P<0.05$, N=6), which was inhibited in the presence of the PKA inhibitor Rp-cAMPS (100 μmol/L, #$P<0.05$, 8-Br-cAMP+Rp-cA vs. 8-Br-cAMP, N=6). Rp-cAMPS alone had no effect (Figure 3B). These results demonstrate that in the absence of PKA or PKG, neither D_1R nor AT_2R can be

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recruited to the cell surface, suggesting important roles for PKA and PKG in D_1R and AT_2R recruitment.

The Importance of PP2A in D_1R and AT_2R Recruitment

We have previously shown that the addition of the D_1R agonist FEN to RPTCs leads to the activation of PP2A[15–18] and AT_2R cell surface recruitment, which are inhibited in the presence of OA. However, we have not tested whether cAMP or cGMP is capable of directly activating PP2A and whether OA is capable of blocking 8-Br-cAMP- and 8-Br-cGMP-dependent D_1R or AT_2R apical plasma membrane recruitment. Figure 4A depicts the amount of phosphate released from the phosphorylated PP2A target peptide. PP2A activity increased by 1.78±0.12-fold (*$P<0.05$ vs. VEH, N=3) following the addition of 8-Br-cAMP. The addition of 8-Br-cGMP also induced a 1.77±0.14-fold increase in PP2A activity (*$P<0.05$, N=3). Either 8-Br-cAMP or 8-Br-cGMP stimulated the recruitment of D_1Rs to the cell surface (1.50±0.08-fold and 1.51±0.09-fold, respectively, *$P<0.05$, N=15) (Figure 4B). Either 8-Br-cAMP or 8-Br-cGMP also stimulated the recruitment of AT_2Rs to the cell surface (1.76±0.05-fold and 1.79±0.08-fold, respectively, *$P<0.05$, N=15) (Figure 4C). Recruitment of either D_1R or AT_2R using cell permeable downstream second messenger agonists 8-Br-cAMP or 8-Br-cGMP was completely blocked by OA (10 nmol/L), a specific PP2A inhibitor.

Na^+ Transport–Na^+ Influx Assay

Both the D_1R and the AT_2R, as well as their respective endogenous agonists, dopamine and Ang III, respectively, have been implicated in inhibition of proximal tubule Na^+ reabsorption that results in natriuresis. Using this cell culture model, we tested whether direct cytoplasmic stimulation with 8-Br-cAMP or 8-Br-cGMP inhibits Na^+ transport and whether PP2A activity is necessary for this inhibition (Figure 5). 8-Br-cAMP (1 mmol/L, 30 min), 8-Br-cGMP (1 mmol/L, 30 min), or both 8-Br-cAMP+8-Br-cGMP significantly inhibited NHE3-mediated Na^+ influx, (34.37±1.48, 27.74±1.58, 31.38±1.24 % reduction vs. VEH, respectively, *$P<0.05$, N=6) with no increase in the effect when combined. The PP2A inhibitor OA (10 nmol/L, 30 min) completely blocked the inhibition of NHE3-mediated Na^+ influx, caused by 8-Br-cAMP, 8-Br-cGMP, or both 8-Br-cAMP+8-Br-cAMP (N=6/group).

Model Depicting Molecular Interactions

The model in Figure 6 summarizes our results. A slice of plasma membrane is depicted with attached microvilli. D_1R and AT_2R previously recruited to the apical plasma membrane appear on the membrane surface. Stimulation by D_1R agonist FEN and the endogenous AT_2R agonist Ang III [inhibited by the D_1R and AT_2R antagonists LE 300 (LE) and PD, respectively] causes an increase in D_1R and AT_2R recruitment to the apical plasma membrane (solid arrows) through a common serine/threonine PP2A-mediated mechanism. PP2A is stimulated via the D_1R stimulation of cAMP production via AC and PKA activation, as well as AT_2R stimulation of cGMP production via GC and PKG activation.

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DISCUSSION

Cell surface membrane recruitment of G protein-coupled receptors from intracellular compartments is crucial to the renewal of response to autocrine and paracrine agonist stimulation. We have presented evidence that the D_1R- and AT_2R-mediated inhibition of RPTC Na^+ transport are linked in order to assure redundancy in Na^+ transport regulation. The cellular messengers associated with these receptors, PKA and PKG, are essential for D_1R and AT_2R recruitment through downstream activation via cAMP and cGMP.

Previous studies demonstrated that the D_1R inhibition of renal Na^+ transport depends on activation of the AT_2R[10,21], Simultaneous stimulation of D_1R and AT_2R induces an increase in cGMP[32]. D_1R stimulation can also induce the recruitment of not only D_1R, but also AT_2R to the apical brush border membrane of human RPTCs[14]. However, cAMP, cGMP, or AT_2R stimulated D_1R translocation has not been reported. In the current study cell permeant cAMP and cGMP were able to induce both D_1R and AT_2R recruitment. The use of both compounds simultaneously does not synergistically or additively increase D_1R and AT_2R recruitment to the plasma membrane. The dose chosen for both 8-Br-cAMP and 8-Br-cGMP is the minimum dose that shows maximal translocation of these receptors and may have depleted the limited pool of internalized receptors. Alternatively the doses used could have maximally stimulated a common saturable shared pathway like PP2A. The selectivity of each cyclic nucleotide is quite high because of the relative affinities to their respective kinases, and the dose responses curves show that a dose of one tenth the chosen dose does not significantly activate recruitment of either receptor, suggesting that direct cross-activation is unlikely. The interdependence of these pathways is supported by the fact that Ang III stimulation of the AT_2R is also uncoupled to cGMP in the SHR[33]. The D_1R is also uncoupled from cAMP, in the SHR and Dahl salt-sensitive rat[34,35]. Future studies will focus on the role of dietary Na^+ on the synthesis and release of dopamine relative to that of conversion of Ang II to Ang III.

The shared molecular mechanism how PKA or PKG activation of PP2A leads to translocation of D_1R and AT_2R is unknown. G Protein-Coupled Receptor Kinase 4 (GRK4) is known to phosphorylate and inactivate the D_1R and may be involved in a particular inherited form of human hypertension[5]. β-Arrestin2 is involved with internalization and inactivation of the D_1R[36]. We hypothesize that PP2A dephosphorylates the D_1R, releasing the receptor from engagement with this well studied internalization mechanism. We have previously shown that the D_1R and AT_2R are found together by protein-protein interaction[14], and perhaps are translocated together when released from internal pools.

Renal interstitial cGMP inhibits renal tubule Na^+ reabsorption via PKG, which regulates Na^+ excretion[37,38]. In this study, the inhibition of either PKA or PKG prevents AT_2R and D_1R translocation respectively to the cell surface. This novel finding further extends the receptor-cAMP/cGMP model to include PKA and PKG which are also closely linked and that both PKA and PKG need to be present in order for D_1R and AT_2R to be recruited to the apical plasma membranes. We also blocked PP2A activity to determine if this is where these two pathways converge and become integrated into one signaling mechanism; our previous studies demonstrated the role of PP2A in D_1R signaling[15–18,39]. PKA has been reported to

activate PP2A by phosphorylating its regulatory subunit, B56δ[40]. In the renal proximal tubule B56δ associates with and dephosphorylates NHE3 to facilitate the inhibitory effect of dopamine on NHE3 activity and expression[41]. PP2A has also been reported to be involved in the D_1R-mediated recruitment of Na^+/K^+ATPases from cytoplasmic pools into the basolateral membrane and its subsequent inhibition in human adenocarcinoma cells[39]. We now report the convergence of D_1R- and AT_2R-mediated inhibition of luminal Na^+ transport in human RPTCs at PP2A. This effect of PP2A may be a general phenomenon because we have also reported that the D_2R-mediated inhibition of inflammation is also mediated by PP2A[42].

Perspective:

Previous studies have not determined whether the D_1R or AT_2R acts independently or collectively to inhibit up to 75% of renal Na^+ transport. The phenomenon of linked G protein coupled receptors has not been readily appreciated until recently. Studies by others have demonstrated elaborate interactions such as allosteric interactions between monomer receptors either as pairs, oligomers, and receptor mosaic assemblies. These assemblies can inhibit or enhance each other's activities, or have an absolute dependence on each other to exert their downstream activities, which is the case for the D_1R/AT_2R interaction. Future studies will focus on determining which other proteins are involved in the Na^+ transport inhibition, as we found for PP2A, and whether there is symmetric or asymmetric allosteric modulation involved in their regulation of Na^+ transport.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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NOVELTY AND RELEVANCE

What is new?

We have demonstrated that there is an absolute co-dependency between D_1R and AT_2R to inhibit Na^+ transport despite their differences in second messenger linkage. This paper is the first to show that direct stimulation of PKA and PKG can induce both D_1R and AT_2R recruitment to the cell surface. Inhibition of either PKA or PKG prevents both D_1R and AT_2R recruitment, highlighting the co-dependency of third messengers in these mechanisms. Furthermore, these two interdependent pathways converge and increase the activity of PP2A which has been shown to increase both D_1R and AT_2R receptor activity.

What is relevant?

This study addresses the controversy over whether the D_1R or the AT_2R dominates over the regulation of Na^+ excretion in the human RPTC. These studies pave the way to determine why codependent systems which regulate an important aspect of electrolyte balance are located in the same renal tubule segment.

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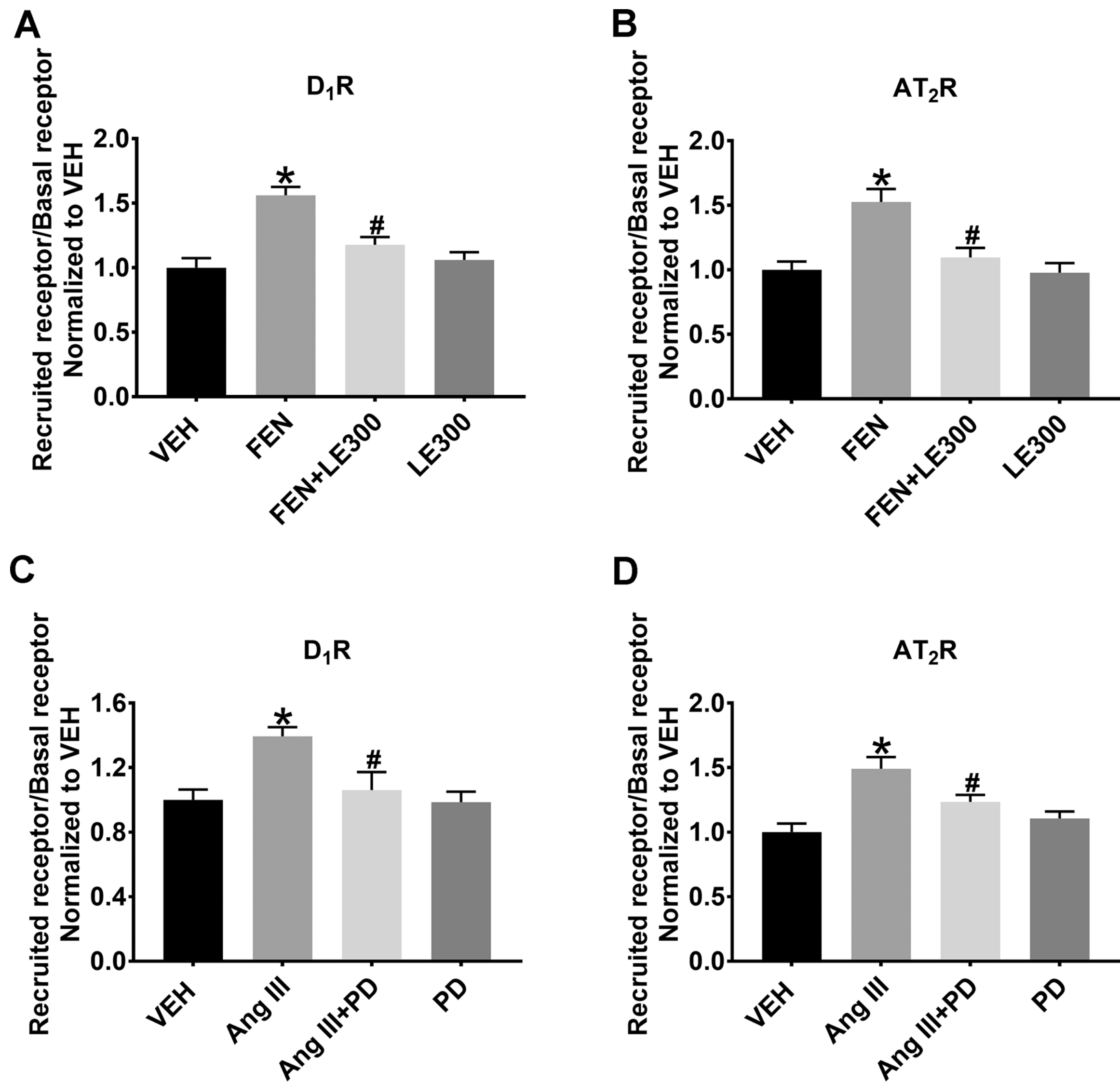

Figure 1: Flow Cytometry Analysis of the Effects of Exogenous FEN and Ang III Treatment on D_1R and AT_2R Recruitment.

FEN (1 μmol/L, 30 min) significantly increases cell surface D_1R (**A**) and AT_2R (**B**) in human RPTCs. *P<0.05 vs. VEH and #P<0.05 vs. FEN. Ang III (10 nmol/L, 30 min) significantly increases cell surface D_1R (**C**) and AT_2R (**D**), respectively. *P<0.05 vs. VEH and #P<0.05 vs. Ang III.

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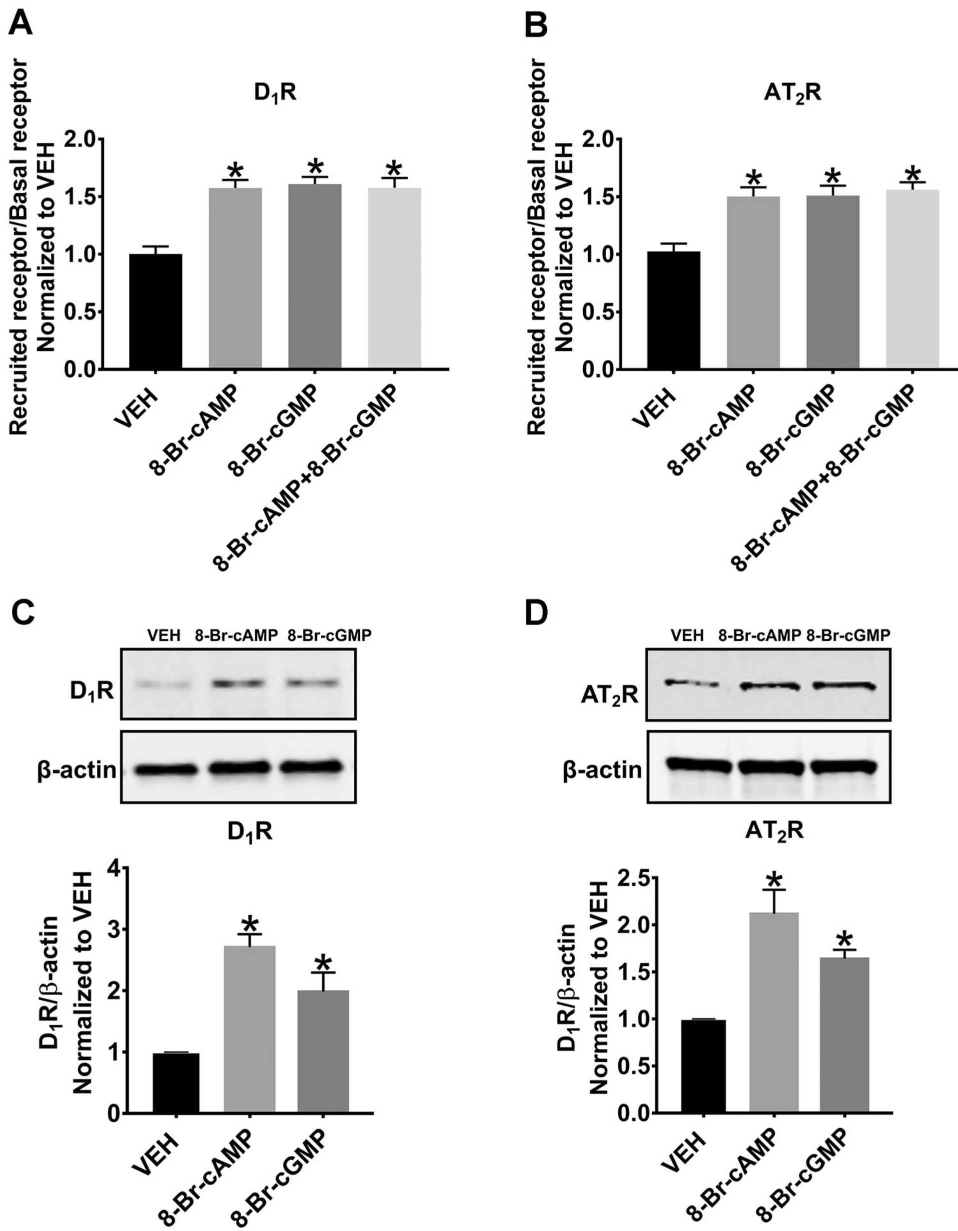

Figure 2: Flow Cytometry and Western Blot Analysis of the Effects of Exogenously 8-Br-cAMP and 8-Br-cGMP Treatment on D_1R and AT_2R Recruitment.

8-Br-cAMP (1 mmol/L, 30 min) or 8-Br-cGMP (1 mmol/L, 30 min) significantly increases cell surface D_1R (**A**) and AT_2R **(B)** in human RPTCs measured by flow cytometry. *P<0.05 vs. VEH. 8-Br-cAMP or 8-Br-cGMP also significantly increases cell surface D_1R **(C)** and AT_2R **(D)** measured by Western blot analysis. All blots are normalized to β-actin and then to VEH. *P<0.05 vs. VEH.

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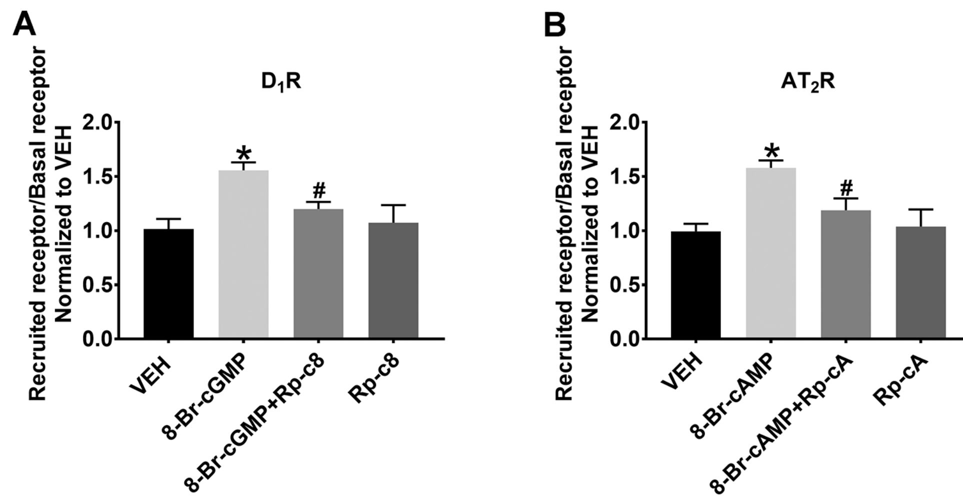

Figure 3. Flow Cytometry Analysis of the Effects of PKG and PKA Inhibition on D_1R and AT_2R Recruitment.

(A). In human RPTCs, 8-Br-cGMP (1 mmol/L, 30 min) recruits D_1R to the cell surface and the PKG inhibitor Rp-8-CPT-cGMPS (Rp-c8; 100 μmol/L) blocks this response. *P<0.05 vs. VEH and #P<0.05 vs. 8-Br-cGMP. **(B).** 8-Br-cAMP (1 mmol/L, 30 min) recruits AT_2R to the cell surface and the PKA inhibitor Rp-cAMPS (Rp-cA; 100 μmol/L) blocks this response. *P<0.05 vs. VEH and #P<0.05 vs. 8-Br-cAMP.

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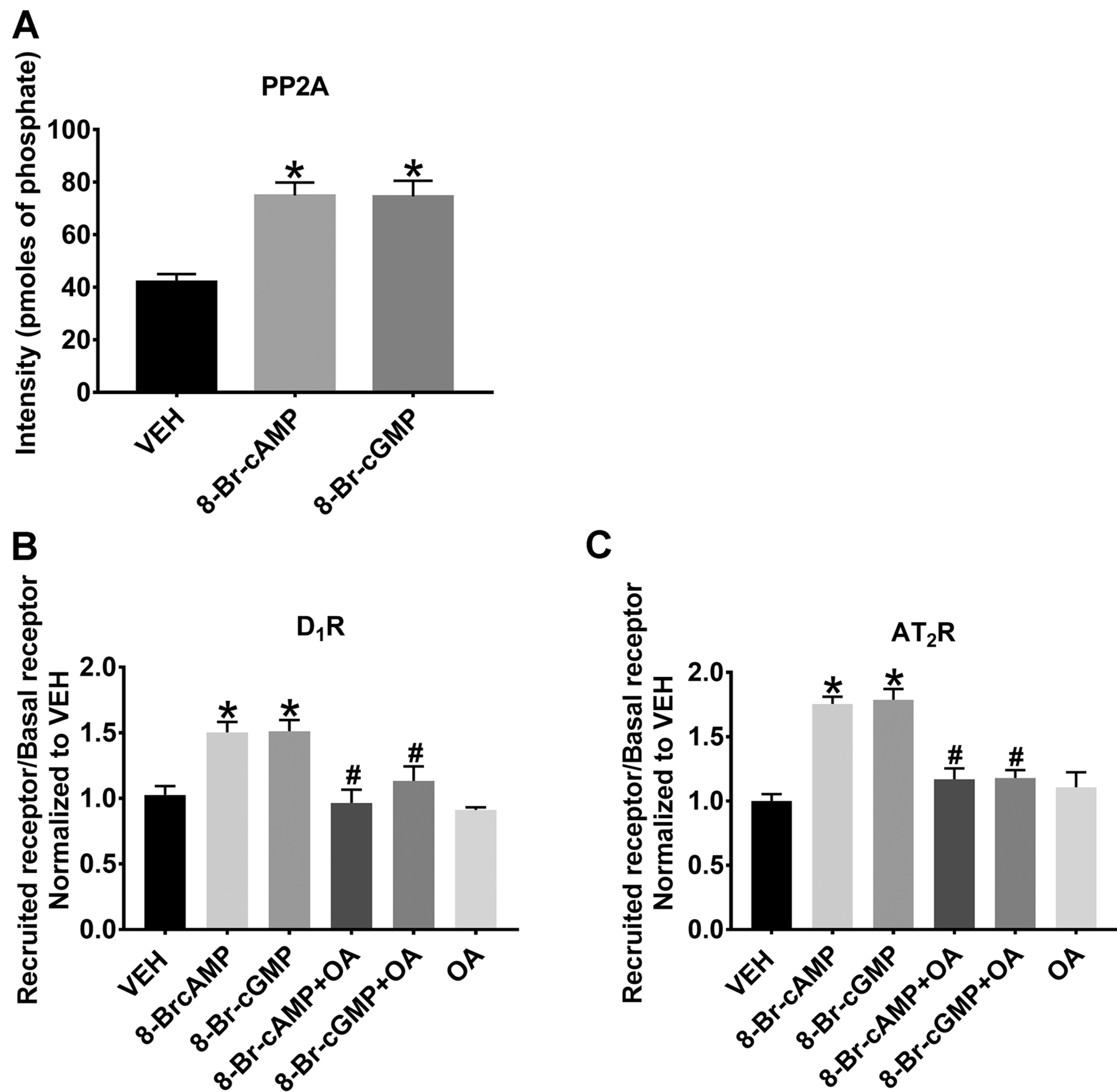

Figure 4: The Importance of PP2A on D_1R and AT_2R Recruitment.

(A). 8-Br-cAMP (1 mmol/L, 30 min) or 8-Br-cGMP (1 mmol/L, 30 min) increases PP2A activity in human RPTCs. *P<0.05 vs VEH. 8-Br-cAMP or 8-Br-cGMP significantly increases cell surface D_1R **(B)** and AT_2R **(C)** recruitment that is blocked with concomitant okadaic acid (OA, a specific PP2A inhibitor; 10 nmol/L) treatment. *P<0.05 vs. VEH and #P<0.05 vs. 8-Br-cAMP or 8-Br-cGMP.

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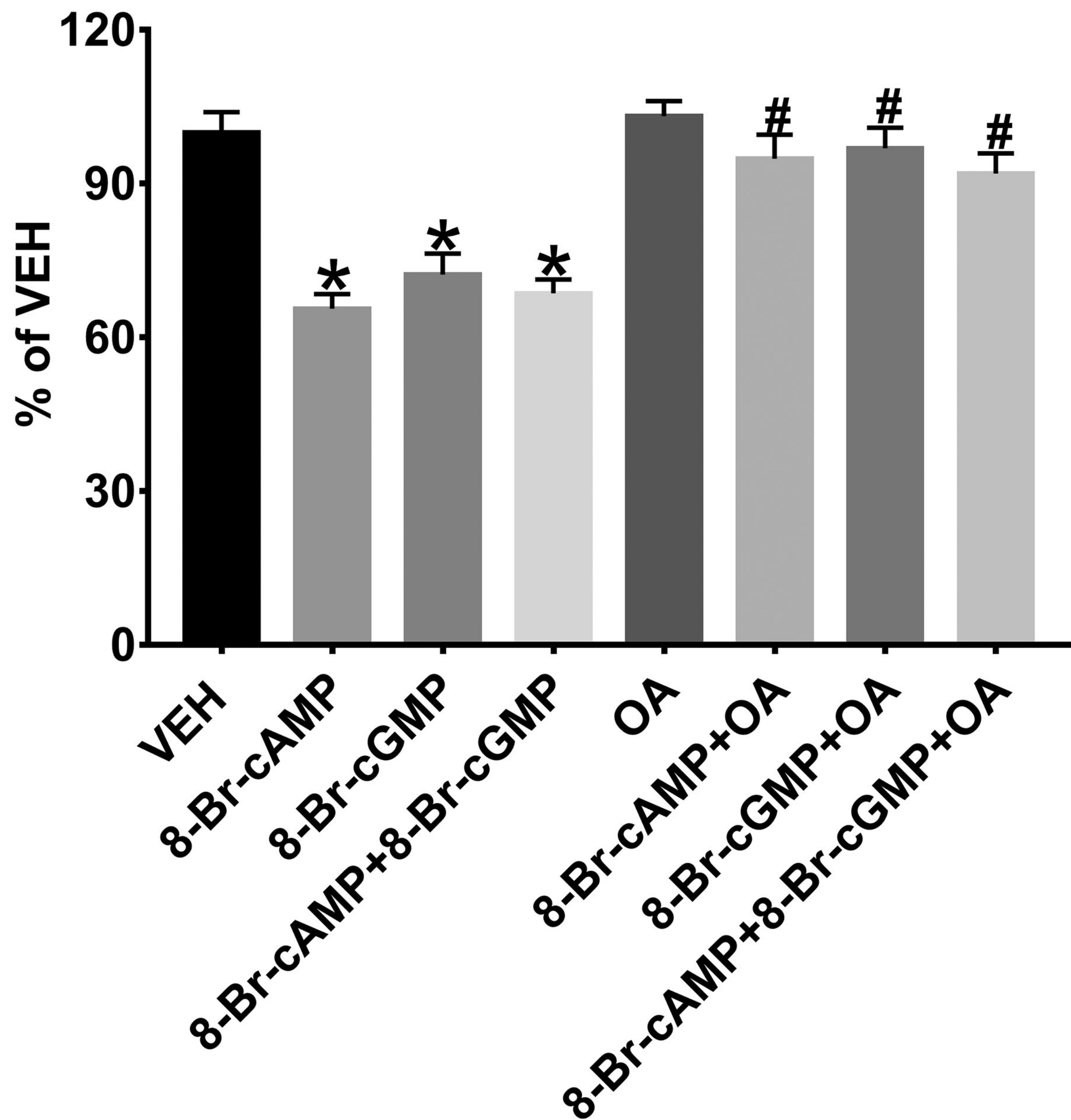

Figure 5: Sodium (Na^+) Transport – Na^+ Influx Assay.

8-Br-cAMP (1 mmol/L, 30 min), 8-Br-cGMP (1 mmol/L, 30 min), or both 8-Br-cAMP+8-Br-cGMP significantly inhibits NHE3-mediated Na^+ influx. *P<0.05 vs. VEH. The PP2A inhibitor okadaic acid (OA, 10 nmol/L) blocks the inhibition of NHE3-mediated Na^+ influx caused by 8-Br-cAMP, 8-Br-cGMP, or 8-Br-cAMP+8-Br-cAMP. #P<0.05 vs. 8-Br-cAMP, 8-Br-cGMP, or 8-Br-cAMP+8-Br-cAMP.

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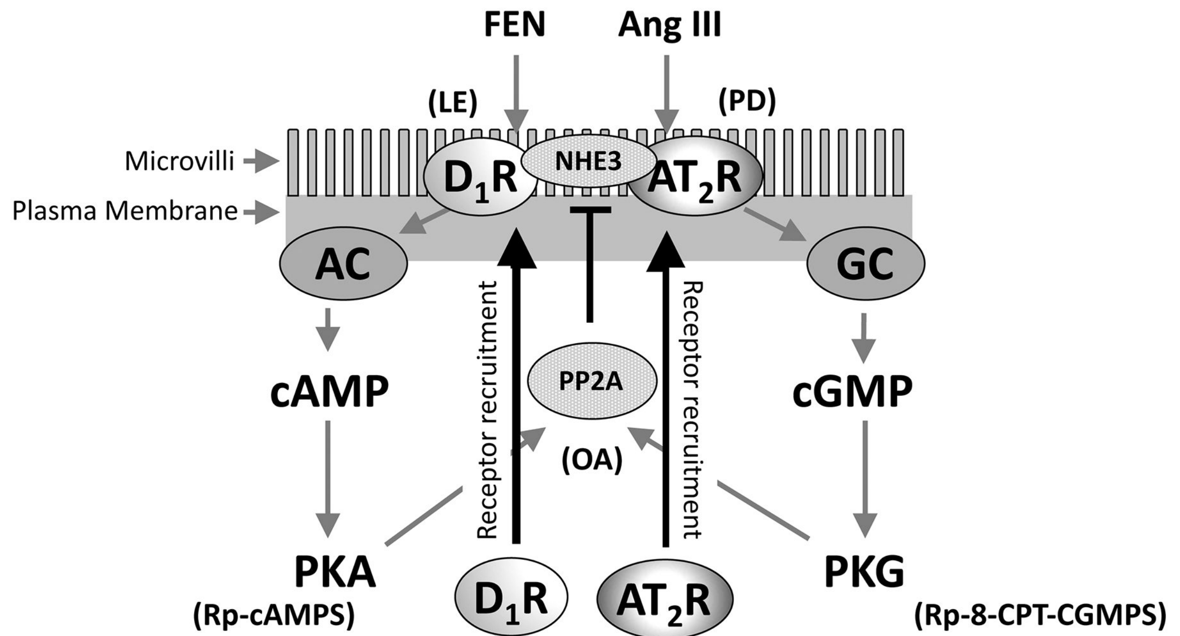

Figure 6: Suggested Model of D_1R and AT_2R Recruitment to the Apical Plasma Membrane of Human RPTCs.

Fenoldopam (FEN), adenylyl cyclase (AC), protein kinase A (PKA), angiotensin III (Ang III), guanylyl cyclase (GC), protein kinase G (PKG), and okadaic acid (OA).