Dopamine and Angiotensin Type 2 Receptors Cooperatively Inhibit Sodium Transport in Human Renal Proximal Tubule Cells

John J. Gildea, Xiaoli Wang, Neema Shah, Hanh Tran, Michael Spinosa, Robert Van Sciver, Midori Sasaki, Junichi Yatabe, Robert M. Carey, Pedro A. Jose, Robin A. Felder

Abstract—Little is known regarding how the kidney shifts from a sodium and water reclaiming state (antinatriuresis) to a state where sodium and water are eliminated (natriuresis). In human renal proximal tubule cells, sodium reabsorption is decreased by the dopamine D1-like receptors (D1R/D3R) and the angiotensin type 2 receptor (AT2R), whereas the angiotensin type 1 receptor increases sodium reabsorption. Aberrant control of these opposing systems is thought to lead to sodium retention and, subsequently, hypertension. We show that D1R/D3R stimulation increased plasma membrane AT2R 4-fold via a D1R-mediated, cAMP-coupled, and protein phosphatase 2A-dependent specific signaling pathway. D1R/D3R stimulation also reduced the ability of angiotensin II to stimulate phospho-extracellular signal–regulated kinase, an effect that was partially reversed by an AT2R antagonist. Fenoldopam did not increase AT2R recruitment in renal proximal tubule cells with D1Rs uncoupled from adenylyl cyclase, suggesting a role of cAMP in mediating these events. D1Rs and AT2R heterodimerized and cooperatively increased cAMP and cGMP production, protein phosphatase 2A activation, sodium-potassium-ATPase internalization, and sodium transport inhibition. These studies shed new light on the regulation of renal sodium transport by the dopaminergic and angiotensin systems and potential new therapeutic targets for selectively treating hypertension. (Hypertension. 2012;60:396-403.) ● Online Data Supplement

Key Words: angiotensin type 2 receptor ▪ dopamine receptors ▪ renal proximal tubule cells ▪ PP2A ▪ cAMP ▪ cGMP ▪ NaKATPase ▪ sodium transport

The kidney is a key organ responsible for regulating sodium and water balance and, ultimately, blood pressure. The intrarenal balance between the natriuretic effect of dopamine and the antinatriuretic effect of angiotensin II (Ang II) is a key factor in whether there is a net increase or decrease in sodium excretion.1 During low or normal sodium intake, basal renal sodium transport is principally regulated by renal Ang II,2 with dopamine playing a relatively minor role.1 Under conditions of moderate excess sodium intake, however, renal dopamine levels increase and inhibit sodium transport.3 Under these conditions, >50% of sodium excretion is attributed to the intrarenal actions of dopamine in dogs4 and rats.5 The natriuretic effect of dopamine is also increased in salt-loaded humans.5 The orchestration of the ultimate effects of Ang II and dopamine results from a complex integration of their corresponding G protein–coupled receptors and intracellular effectors. The D1-like receptors (D1R and D2R in mammals) are the primary dopaminergic receptors involved in sodium transport inhibition. Selective stimulation of the D1R acts through a cAMP-mediated mechanism involving G protein receptor–coupled kinase type 4 (GRK4) to decrease both NHE3, the primary sodium transporter in the brush border (apical) membrane, and NaKATPase, the sodium pump in the basolateral membrane.7,8 Selective stimulation of the D2R inhibits the antinatriuretic activity of the angiotensin type 1 receptor (AT1R).9 Ang II stimulates 2 G protein–coupled receptors in the kidney, the AT1R, which is directly stimulated by Ang II, and the angiotensin type 2 receptor (AT2R),1,10 which is stimulated only after Ang II is converted to Ang III.11 Thus, sodium transport may be increased or decreased depending on the concentration of Ang II and dopamine and the state of sodium balance.1,10,12

There is little information on the autologous and heterologous regulation of angiotensin and dopamine receptors, particularly in humans. It is known that AT2Rs, which are highly expressed in the plasma membrane (PM), respond to Ang II stimulation by a relatively rapid (T1/2=20 seconds) downregulation.13,14 In contrast, D1Rs, which are in relatively low abundance in the PM, respond to dopamine stimulation by a relatively rapid (T1/2=60 seconds) upregulation or recruitment to the PM from the cytoplasm.15-17 D1R and
AT, R can decrease each other’s expression in rodents and humans, whereas the D1R, D2R, and D3R can all physically interact with AT, R to inhibit its function.20–22

AT2Rs are in low abundance in the PM compared with AT1Rs and are not downregulated in response to Ang II stimulation. Little is known about Ang III–stimulated AT2Rs and their transregulation by dopamine receptors in humans. In rodents, D1-like receptor stimulation causes AT2Rs to translocate to the brush border of the rat renal proximal tubule, and the subsequent natriuretic response is blocked by the AT2R antagonist PD-123319 (PD). This suggests that AT2Rs are necessary for D1-like receptor–mediated natriuretic effects. AT2Rs are upregulated after AT1R blockade and, thus, may provide significant counterbalancing effects for AT1Rs.24

The current study tests the hypothesis that stimulation of the D1R upregulates AT2Rs via translocation to the PM, physical heterodimerization with D1Rs, and cooperative functionality. Because inhibitors of posttranscriptional protein expression (small interfering RNA [siRNA] and antisense oligonucleotides) are not 100% effective, we used human renal proximal tubule cells (RPTCs) and AT2Rs to translocate to the brush border of the rat renal proximal tubule, and the subsequent natriuretic response is blocked by the AT2R antagonist PD-123319 (PD).17 This suggests that AT2Rs are necessary for D1-like receptor–mediated natriuretic effects. AT2Rs are upregulated after AT1R blockade and, thus, may provide significant counterbalancing effects for AT2Rs.24

Materials and Methods

Cell Lines
Human kidneys from fresh surgical specimens came from patients who had unilateral nephrectomy attributed to renal carcinoma or trauma. A university institutional review board–approved protocol was used according to the Declaration of Helsinki, using the recent version of Title 45, Part 46, US Code of Federal Regulations. This study used the same 2 cell lines characterized extensively in a previous study (nRPTCs, line i22 and uRPTCs, line i19). Details about these cells and positive control HEK-293 cells (stably transfected with human D1R and AT2R cDNA) are listed in the online-only Data Supplement.

PM Protein Expression
Detergent-free cell surface membrane sheets (PM) were isolated as reported previously and outlined in the online-only Data Supplement. Briefly, RPTCs were biotinylated with the amine-reactive cleavable biotin labeling reagent (sulfo-NHS-S-S-biotin) and lysed. Biotinylated membranes were isolated with streptavidin Sepharose beads. The effects of the dopamine-1 like agonist, fenoldopam (FEN) or dimethyl sulfoxide vehicle control (VEH) on D1R, D2R, and AT2R protein levels were evaluated using a multiblot multitransfer method.

D1R and AT2R Coimmunoprecipitation
nRPTCs and HEK-293 lysates were immunoprecipitated with a rabbit D1R antibody (Santa Cruz H109) and detected using D1R rat monoclonal (Sigma D2944) and AT2R goat polyclonal (Santa Cruz N19) antibodies, as described in the online-only Data Supplement. Endogenous D1R and AT2R protein-protein interaction was confirmed by immunoprecipitating AT2R with 4 μg of rabbit AT2R antibody (Santa Cruz H143), with subsequent detection using the same 2 antibodies used for D1R immunoprecipitation.

Sensitized Emission Forster Resonance Energy Transfer Microscopy
Forster resonance energy transfer (FRET) Microscopy, a technique used to determine whether two spectrally overlapping fluorophores are within 10 nm proximity to one another, was performed on fixed nonpermeabilized nRPTCs using extracellular epitope-specific and directly, fluorescently labeled antibodies to D1R and AT2R (Alexa 488 and 555, respectively). Details are in the online-only Data Supplement.

Ang II Peptide Binding
RPTCs were stimulated (30 minutes) with phosphate buffered saline vehicle control, FEN, or FEN plus LE300, as described in the online-only Data Supplement. Briefly, cells were cooled and incubated (1 hour) with fluorescently labeled Ang II in the presence of losartan (LOS), PD, or a combination of LOS+PD. Ang II+PD represents total AT2R binding and Ang II+LOS represents total AT2R receptor binding.

ICUE3 and cGMP FRET Biosensors
RPTCs were transfected with 4 μg of each biosensor using a Gene Pulser Mx Cell 96-well electroporation system (Bio-Rad), as reported previously and in the online-only Data Supplement. Cyan fluorescent protein (CFP) excitation and emission divided by CFP excitation and yellow fluorescent protein (YFP) emission ratiometric imaging on a 96-well Olympus IX81 automated confocal microscope was performed over 20 minutes, as described previously.

Protein Phosphatase 2A Assay
RPTCs at 80% confluence were serum starved and exposed to agonists (30 minutes). Cells were lysed according to kit instructions (Millipore), with details in the online-only Data Supplement.

Phospho-Extracellular Signal–Regulated Kinase Measurement
nRPTCs and uRPTCs were cultured, serum starved, and exposed to vehicle control or FEN (30 minutes) before the addition of Ang II or antagonists. Cell lysates were loaded onto nitrocellulose in a 96-well dot-blot apparatus, as described in the online-only Data Supplement.

Total Internal Reflectance Microscopy
Monesin was added (30 minutes) to serum-starved nRPTCs followed by FEN or FEN+Ang III (additional 30 minutes). Cells were fixed, blocked, and stained with NaKATPase-α subunit antibody using an Alexa-488–labeled donkey antimouse secondary antibody for detection. Imaging details are in the online-only Data Supplement.

siRNA to GRK4, D1R, and D5R
GRK4 siRNA (50 nmol/L) or scrambled control was transfected in siRNA to GRK4, D1R, and D5R

NaKATPase-Mediated Sodium Efflux
NaKATPase activity was measured as ouabain-sensitive intracellular sodium efflux, using an intracellular sodium sensitive dye, sodium-binding benzofuran isophthalate, as described previously and in the online-only Data Supplement. Briefly, RPTCs were labeled with sodium-binding benzofuran isophthalate and incubated in potassium-free medium followed by incubations with FEN, Ang III, or both. To initiate sodium efflux, 5-(N-ethyl-N-isopropyl) amiloride and potassium were added to simultaneously block sodium influx and allow NaKATPase to begin active sodium transport. Time-lapse ratio imaging was conducted using an automated confocal microscope.
Statistics
The data are expressed as mean ± SE. Comparisons within and among ≥3 groups were made by repeated-measures or factorial ANOVA, respectively, followed by the Student-Newman-Keuls or Duncan test. A t test was used for 2-group comparisons. A value of P < 0.05 was considered significant.

Results
Simultaneous Western “multiblot” analysis of RPTC protein expression compared the relative amounts of PM receptor expression, minimizing interexperiment variability (Figure 1). In nRPTCs, FEN (1 μmol/L, 30 minutes) increased PM expression of D1R (as published7) and AT2R (49.9 ± 35.2% of control), whereas D5R expression was unaffected (Figure 1A through 1C). FEN decreased AT1R expression in nRPTCs and uRPTCs. Basal AT1R expression was higher in uRPTCs than in nRPTCs (28.0 ± 0.06% over nRPTC; *P < 0.05; n = 4).

We established time-course and concentration-response curves for the D1R-mediated recruitment of AT2R (Figure 2). FEN stimulated a 3.96 ± 0.17-fold increase in AT2R PM recruitment (10 minutes; Figure 2A) but only in nRPTCs. A maximal response continued for 30 minutes and declined at 60 minutes. The D1-like receptor antagonist SCH 23390 blocked the 30-minute AT2R recruitment induced by FEN.

Concentration-dependent FEN-stimulated responses were measured at 30 minutes in RPTCs (Figure 2B). FEN significantly stimulated AT2R PM recruitment at 100 nmol/L (EC50 of 29.2 ± 3.2 nmol/L; n = 4) but only in nRPTCs. Both SCH 23390 (5 μmol/L) and okadaic acid (protein phosphatase 2A [PP2A] inhibitor, 10 nmol/L) blocked the FEN effect (neither SCH 23390 nor okadaic acid alone had an effect). Forskolin (adenylyl cyclase agonist, 10 μmol/L) increased AT2R 4-fold in nRPTCs and uRPTCs (n = 4; #P < 0.05 versus vehicle control).

The next 2 panels show siRNA studies: Figure 2C shows FEN stimulation of uRPTCs, which have impaired membrane recruitment of the D1R because of increased GRK4 activ-
scrambled FEN did not affect AT2R PM expression in siRNA. Silencing the expression of both D1R and D5R did not decrease by D1R siRNA by 84.3% /11006 after FEN stimulation (5.0 /11006 14.0%). This is similar to our previous reports that GRK4 siRNA rescued the FEN-PM expression by 92.9% /11006 70.6% /11006 0.05 vs control [VEH]). A and B, ■, nRPTC; ■, uncoupled RPTC (uRPTC). C, Dopamine-1 receptor (D1R) is recoupled after silencing of G protein receptor–coupled kinase type 4 (GRK4), allowing FEN-induced AT2R recruitment in uRPTCs (n=3; *P<0.05 vs others). One representative immunoblot is displayed. D, FEN increased AT2R PM expression in nRPTCs, which is reversed with small interfering (si)RNA silencing of the D1R (third bar) gene (not the D5R, fourth bar). Silencing the expression of both D1R and D5R (fifth bar) was the same as D1R siRNA, indicating that D1R works without D5R to recruit PM AT2Rs.

Figure 2D shows siRNA in nRPTCs to separate out the roles of the 2 D1-like receptors. AT2R membrane recruitment after FEN stimulation (5.0±0.3-fold versus control) was decreased by D1R siRNA by 83.5±5.9% but not by D5R siRNA. Silencing the expression of both D1R and D5R did not alter the magnitude of the D1R siRNA response, indicating that the D1R works without the D5R to recruit PM AT2Rs. Neither D1R nor D5R scrambled oligonucleotide controls had any effect on AT2R PM expression (data not shown). We reproduced these findings using antisense (same D1R and D2R antisense and scrambled oligonucleotide controls, as published previously5; data not shown).

We performed coimmunoprecipitation to investigate a physical association between D1Rs and AT2Rs (Figure S1, available in the online-only Data Supplement). In Figure S1A, nRPTC lysates were immunoprecipitated with rabbit anti-AT2R antibody or nonspecific IgG and analyzed by Western blot. Both D1R and AT2R were detected in the AT2R immunoprecipitate but not in the nonspecific IgG immunoprecipitate. In Figure S1B, a rabbit anti-D1R antibody was substituted for the AT2R immunoprecipitating antibody used in Figure S1A, producing similar results. The same procedures as used in Figure S1B were repeated on lysates from D1R and AT2R stably transfected HEK-293 cells, and a similar result was found (Figure S1C). This suggests that the D1R/AT2R association is not cell-type–specific, as long as both proteins are found within the same cell.

We examined D1R/AT2R colocalization in the PM using fluorescence microscopy of labeled extracellular epitope-specific antibodies (Figure S1D). Cell surface D1R and AT2R were found colocalized in live nRPTCs using fluorescently labeled extracellular epitope-specific antibodies. An Alexa-555–labeled D1R polyclonal antibody and an Alexa-488–
Ang II decreased PP2A activity, whereas Ang III had no effect. FEN caused an increase in PP2A activity but only in nRPTCs. Forskolin served as a positive control for the stimulation of cAMP production. The forskolin-stimulated increase in PP2A activity agrees with previous reports that the uncoupling of the D1R is proximal to its coupling to adenylyl cyclase.26,28

PP2A Activity (% of VEH)

PM AT2Rs are in low abundance in basal conditions. Thus, we examined the relative abundance of AT2R and AT1R before and after FEN stimulation (Figure S2). We used selective antagonists to measure Ang II-649 binding to AT1Rs (Ang II+PD) or AT2Rs (Ang II+LOS). Nonspecific binding was determined by measuring Ang II binding in the presence of PD+LOS and it approximated autofluorescent levels. With FEN stimulation, AT1R levels decreased in both nRPTCs and uRPTCs. This effect was blocked by LE300 (D1R/D5R antagonist), which agrees with previous reports that AT1R is mediated by a D5R-selective pathway and is not different between nRPTCs and uRPTCs,9 and GRK4 does not desensitize the D3R.1,18 The AT1R peptide binding was initially 4-fold lower than AT1R density, but when stimulated with FEN, the AT1R peptide binding became almost equal to basal AT1R expression in nRPTCs. Again, LE300 blocked this effect. In uRPTCs, there was no equalization of the 2 receptor types.

AT1R stimulates phosphorylation of p44 and p42 mitogen-activated protein kinase (phospho-extracellular signal–regu-
lated kinase [pERK]1/2) and AT1R inhibits pERK1/2 and AT2R. Because peptides in short-term stimulation are unable to penetrate the outer membrane of cells, we used pERK1/2 as a relative functional readout of cell surface AT1R and AT2R stimulation (Figure S3). In the basal state, the addition of Ang II to nRPTCs or uRPTCs caused a 4-fold increase in pERK1/2 that was completely inhibited by the AT1R inhibitor LOS but not the AT2R inhibitor PD. The addition of FEN decreased pERK1/2 in nRPTCs. FEN addition before Ang II stimulation dramatically changed the nRPTC response to Ang II in a manner consistent with the changes in cell surface expression seen in Figure S2. Ang II stimulation of pERK1/2 was dramatically reduced after the nRPTCs were stimulated with FEN but less so in uRPTCs. Coadministration of LOS further reduced pERK1/2, an indication of residual AT1R on the cell surface. In nRPTCs, PD’s reversal of the inhibition shows that newly recruited AT2Rs were partially responsible for the change in responsiveness to Ang II, but in uRPTCs no reversal was seen.

The sodium pump NaKATPase is one of the principal regulators of sodium transport. We, therefore, examined the effect of Ang III and/or FEN on NaKATPase expression in the PM (Figure 5) via total internal reflectance microscopy (Figure 5A) and also measured ouabain-sensitive sodium transport (Figure 5B). In Figure 5A, the NaKATPase basolateral membrane expression was measured in nRPTCs using mouse anti–NaKATPase-α and imaged at 70 nm above the coverslip surface. NaKATPase internalization was seen as a loss of total internal reflectance fluorescence intensity. In Figure 5B, neither Ang III nor FEN (FEN at suboptimal concentration of 100 nmol/L) decreased sodium efflux. However, the combination of FEN and Ang III significantly reduced sodium efflux. This effect was blocked by the AT1R antagonist PD, signifying a synergistic inhibition of NaKATPase by D1R and AT2R.

Figure S4 shows a model depicting the known intracellular pathways that are involved with dopaminergic reduction of sodium transport in a renal proximal tubule cell. Figure S5 shows a model of AT1R signaling to pERK1/2 and D1R/DSR and AT2R effects on this signaling in nRPTCs and uRPTCs.

Discussion

Trafficking and downregulation are 2 key regulatory mechanisms in the regulation of cell surface expression of G protein–coupled receptors. In cell models like HEK-293 with overexpressed receptors, AT1Rs rapidly desensitize and internalize after Ang II stimulation, whereas AT2Rs do not. Endogenous AT1Rs in rodent and in human RPTCs (current data) appear to behave differently. Our studies demonstrate that D1R/D2R stimulation caused a recruitment of AT2Rs to the PM of human RPTCs, similar to the effect of D1R/D2R stimulation on D1R recruitment. Using selective siRNA, we demonstrated that the receptor responsible was the D1R and not the D2R. D1R stimulation recruited PM AT2Rs from very low basal levels to levels comparable to AT1Rs. This AT2R recruitment was not seen in uRPTCs, which have a D1R that is uncoupled from its effector proteins.

The D1R-mediated recruitment of AT2R in nRPTCs was >4-fold with a T1/2 of 5 minutes, which is consistent with the relatively rapid trafficking of G protein–coupled receptors after agonist stimulation. The EC50 for the D1R-mediated recruitment of AT2R was in the high nanomolar range, which is in agreement with the affinity of D1-like receptors in the renal proximal tubule. Maximal recruitment of AT2R to the PM occurred at 10 minutes and declined at 60 minutes, implying D1R desensitization between 30 and 60 minutes. These data demonstrate that AT2R cell surface translocation is necessary for its various functions, one of which is to inhibit AT1R signaling to pERK 1/2 (a surrogate marker for AT1R function). By inhibiting the cell surface–translocated AT2R with the PD compound, the inhibitory influence
of AT1R is reversed, and AT1R can then signal more strongly to pERK1/2. uRPTCs offer a unique model of D1R dysfunction because the D1R is uncoupled from effector proteins and adenylyl cyclase.1,26,35,36 In previous studies, we demonstrated that inhibition of GRK4×3 methods (intracellular introduction of heparin, which inhibits all GRK isoforms; selective inhibition of GRK4 by GRK4 antisense oligonucleotides; and GRK4-specific siRNA) all blocked the constitutively increased activity of GRK4 in uRPTCs and, thus, restored D1R coupling to specific siRNA) all blocked the constitutively increased activity of GRK4 by GRK4 antisense oligonucleotides; and GRK4-heparin, which inhibits all GRK isoforms; selective inhibition of GRK4 function.1,12,26,28,30,37,38 The current report shows similar findings for AT1R-Rs, suggesting a key role for GRK4 because it has been shown to directly regulate D1R and D3R function.1,12,26,28,30,37,38

The current studies extend our previous observations that D1R stimulation reduces AT1R function and expression in rodents18 and humans,9 and D1R stimulation increases PM D1R and AT1R expression while reducing AT1R.31 In the current studies, D1R/D3R stimulation reduced Ang II–stimulated pERK expression by >80%. Nearly 50% of this signal was reversed by AT1R blockade, suggesting that the other 50% of the inhibition was mediated by D1R inhibition of AT1R.

We have determined that D1R stimulation of AT1R recruitment is cAMP and PP2A (specific inhibitor, okadaic acid at 10 nmol/L) dependent. D1R-like receptor stimulation increases PP2A activity in rodents but only in Wistar-Kyoto and not spontaneously hypertensive rat cells.37 Here we extend these studies to demonstrate that, in humans, Ang II inhibits PP2A activity, whereas Ang III does not. This can in part be explained by Ang III having a 5-fold increase in affinity for the AT1R over the AT1R.39 FEN prestimulation shifts the cell sensitivity to an Ang III–mediated increase in sodium excretion from its normally antinatriuretic state under conditions of a moderate sodium load. The relatively high concentrations of Ang II in low or normal sodium load conditions would stimulate the relatively high levels of AT1R, favoring sodium reabsorption. On dopaminergic stimulation with moderate sodium load, the AT1R would be downregulated/deactivated and the natriuretic AT2R, D1R, D3R, and D5R would be upregulated/activated, leading to a net increase in sodium excretion. Our data suggest that use of an AT1R agonist and a method to restore D1R coupling by inhibiting GRK4 expression or providing an increase in proximal tubule cAMP may provide selective antihypertensive therapy. Similarly, the combination of a D1R agonist and an AT1R antagonist may provide an alternative therapeutic approach.

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Disclosures

None.

References

Novelty and Significance

What Is New?
- D1R, but not the D2R, stimulation causes AT2R translocation to the cell surface of human renal proximal tubule cells
- AT1R translocation is inhibited by overactive GRK4
- PP2A activation is necessary for AT1R translocation
- The D1R and AT2R heterodimerize
- The D1R and AT2R functionally cooperate in cAMP and cGMP production and inhibition of sodium transport.

What Is Relevant?
- Either overactive GRK4 or underactive PP2A could lead to decreased surface localization and function of both D1R and AT2R leading to hypertension
- Novel compounds that may inhibit GRK4 and or activate PP2A might be used in conjunction with D1R and AT2R agonists in the treatment of hypertension.

Summary
This article is the first to show how the dopaminergic system shifts the renal proximal tubule from a sodium-retaining state to a sodium-eliminating state. This shift is mediated by the simultaneous stimulation of the AT2R (via Ang II) and dopamine receptors, thus unifying the activity of these pathways that were thought previously to be independent. We feel that this article demonstrates how defects in either of these 2 pathways are relevant to the etiologies of hypertension.
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Short Title: The Renal Sodium Switch

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Expanded Materials and Methods

Cell lines:
Renal proximal tubule cells (RPTCs) were isolated from human kidney specimens from patients who had unilateral nephrectomy due to renal carcinoma or trauma. Only the visually and histologically normal pole, distal from the affected part of the kidney, was used to isolate RPTCs. Culture medium used for propagating RPTCs was: DMEM/F12 with 2% fetal bovine serum, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenium, 36 ng/mL dexamethasone, 2 ng/mL triiodothyronine, 10 ng/mL human recombinant epidermal growth factor (Sigma), 100 U/mL penicillin G, 100 μg/mL streptomycin, and 2.5 μg/ml plasmocin.

D1R coupled and uncoupled cells: We have identified two distinct sub-populations of RPTCs, those that have D1Rs with normal adenylyl cyclase coupling (nRPTCs) and those whose D1R coupling to adenylyl cyclase is defective (uncoupled, or uRPTCs) as the consequence of aberrant GRK4 activity. The characterization of this uncoupling phenomenon has been described in detail in our previous papers, especially Gildea et al, 2010. The majority of experiments described below were done with both types of cell lines. Certain experiments involving just nRPTC (e.g. looking at “normal” receptor association) or uRPTC (siRNA experiments trying to “fix” the defect) are noted as such.

HEK-293 cells stably transfected with human D1R cDNA were used as positive controls in several experiments. HEK-293 stably transfected with human D1R cDNA (Yu et al., 2004) were also transfected with human AT2R (Missouri S&T cDNA Resource Center, Cat# AGTR200000) and selected with 200 μg/ml G418. AT2R expression was confirmed by live cell incubation with a fluorescently labeled AT2R antibody, using HEK-293 cells that heterologously express only D1R as a control.

Agonists/Antagonists: The agonists FEN, Ang II and Ang III were all purchased from Sigma. SCH 23390 (SCH, Sigma) and LE300 (LE, Tocris) were used as D1R/D5R antagonists. The AT2R antagonist PD123319 (PD), the AT1R antagonist losartan (LOS) and the adenylyl cyclase agonist forskolin (FSK) were all purchased from Sigma.

Measurement of plasma membrane protein expression:
nRPTCs and uRPTCs were biotinylated with the amine-reactive, cleavable biotin labeling reagent (sulfo-NHS-S-S-biotin) (Vickery and von Zastrow, 1999). The cells were scraped and lysed in PBS with Halt Protease Inhibitor Cocktail (Pierce). Biotinylated membranes were isolated with streptavidin sepharose beads, washed, and then released along with biotin-labeled proteins in boiling western gel loading buffer with reducing agent. In order to make direct comparisons of the effects of fenoldopam (FEN, 1 μmol/L, 30 min) with DMSO vehicle control (VEH) on the D1R, D5R and AT2R protein levels, western blotting was performed using a multi-blot multi-transfer method (Multi-Blot Kit, Kodak). Western blot membranes were incubated with antibodies specific to AT2R (1:200 dilution, Santa Cruz 9040), D1R (1:200 dilution, Santa Cruz 14001), and D5R (1:500 dilution, Santa Cruz 25650). Equal loading was verified by Ponceau-S staining and blotting with monoclonal antibody to β-tubulin (0.1 μg/ml, Developmental Studies Hybridoma Bank, clone E7).
Concentration and time response for AT2R expression:
nRPTCs and uRPTCs were incubated with varying concentrations of the D1R/D5R receptor agonist, fenoldopam (10^{-8} to 10^{-5} mol/L) and/or the D1R/D5R receptor antagonist SCH-23390 (5 µmol/L). The time course (0, 5, 10, 15, 30, and 60 min) was studied using a FEN concentration that produced the maximum response (1 µmol/L). AT2Rs from the plasma membrane were isolated using the procedure described above and transferred to a dot-blot apparatus (Bio-Rad). Western dot blotting was performed using rabbit AT2R antibody (1:200, Santa Cruz, H143) in triplicate. All AT2R dot-blot experiments were done with one lot of AT2R antibody (Lot #H201) because this particular antibody lot produced a single band by western blotting that was completely blocked by polypeptide inhibition. SCH-23390 (5 µmol/L), okadaic acid (OA, PP2A inhibitor, 10 nmol/L) and forskolin (FSK, adenylyl cyclase agonist, 10 µmol/L) were used.

Association of D1R and AT2R by co-immunoprecipitation:
nRPTCs were incubated with VEH or FEN (1 µmol/L) for 30 min. Lysates were made with M-PER (Mammalian Protein Extraction Reagent, Thermo Scientific) containing Halt Phosphatase and Protease Inhibitor Cocktail (Pierce). Proteins were quantitated by BCA (Pierce). 500 µg of protein lysate were immunoprecipitated with 5 µg of rabbit D1R antibody (Santa Cruz H109) by incubating overnight at 4°C. Non-specific rabbit IgG was used as a negative control. The next day, lysates were incubated with Protein A/G agarose (Pierce) for 90 min. Beads were washed and protein was eluted with 50 µL of loading buffer, heated to 65°C for 5 min, placed on an AnyKd GTX gel (Bio-Rad) and transferred to nitrocellulose. For western blotting, a D1R rat monoclonal (Sigma D2944, 1:500 dilution) and an AT2R goat polyclonal (Santa Cruz N19, 1:200 dilution) were used. Secondary antibodies used were donkey anti-rat 800 (LI-COR) and donkey anti-goat 680 (LI-COR) and both proteins were measured simultaneously on an Odyssey Near Infrared imaging system (LI-COR).

HEK-293 cells that were stably transfected with D1R and AT2R were also tested for D1R and AT2R co-immunoprecipitation using the same procedure and antibodies. Endogenous D1R and AT2R protein-protein interaction was also confirmed by immunoprecipitating AT2R with 4 µg rabbit anti-AT2R antibody (Santa Cruz, H143), with subsequent detection using the same two antibodies used for D1R immunoprecipitation.

Sensitized emission FRET microscopy:
Fixed non-permeabilized nRPTCs were analyzed using extracellular epitope-specific and directly, fluorescently-labeled antibodies to the D1R and AT2R using Alexa 488 and Alexa 555 dyes, respectively. Positive (ICUE-YR cAMP FRET Biosensor) and negative controls (each single antibody alone, as well as Alexa 488-labeled D1R with an Alexa 555-labeled monoclonal antibody to the transferrin receptor) verified that the microscope setup and calculations were working properly. The specifics of the staining procedure were: cells were plated on collagen IV-coated glass bottom 96-well plates (Matrical) to reach 30% confluence the next day, then serum- and growth factor-starved overnight. The cells were washed twice with phosphate-buffered saline (PBS) and lightly fixed in PBS containing 1% paraformaldehyde for 2 min. They were next washed with Tris-buffered saline (TBS) 3 times for 5 min to inactivate the residual crosslinking agent. The fixed cells were blocked overnight in PBS containing 5% BSA in PBS. All incubations were done for 1 hr in PBS, 1% BSA at a 1:100 dilution with gentle rocking at room temperature, followed by washing three times (5 min each) in PBS.
The cells were imaged using an Olympus IX81 automated multi-well spinning disk confocal microscope. Images were captured under identical conditions and the automated spectral bleed through calculations performed according to the Slidebook 4.2 Fret Module macro. The filters used were in a Sedat configuration with Alexa 488-labeled D1R (excitation filter 485/20 nm – emission filter 525/30 nm), Alexa 555-labeled AT2R (excitation filter 560/25 nm – emission filter 607/36 nm), and FRET channel (excitation filter 485/20 nm – emission filter 607/36 nm). The spectral bleedthrough was automatically subtracted using the bleedthrough correction FRET macro. Positive controls for specific fluorescent antibody binding were D1R-stably transfected HEK-293 cells that also had been stably transfected with AT2R, and negative controls were non-transfected HEK-293 cells.

Ang II peptide binding:
nRPTCs and uRPTCs were cultured in glass-bottomed 96-well plates, serum-starved overnight, then stimulated (30-min) with VEH, FEN (1 µmol/L), or FEN plus the D1R/D5R antagonist LE300 (LE, 10 µmol/L). Cells were cooled on ice, incubated (1-hr) with 1 µmol/L Dylight 649 fluorescently-labeled Ang II (Alta Biosciences) in the presence of the AT1R antagonist losartan (LOS, 10 µmol/L), the AT2R antagonist PD123319 (PD, 10 µmol/L) or a combination of LOS+PD. Cells were washed and imaged on an Odyssey Near Infrared imager (LI-COR). Ang II+PD represent total AT1R binding and Ang II+LOS represent total AT2R receptor binding.

ICUE3 and cGMP measurements using FRET biosensors:
Transfection of RPTCs with 4 µg/mL of each biosensor was performed using a Gene Pulser Mx Cell 96 well electroporation system (Bio-Rad). The cAMP FRET biosensor pcDNA3-ICUE3 and the cGMP biosensor pcDNA3.1-Cygnet2.1 (Addgene plasmid 19737)-transfected cells were plated in collagen-coated 96 well plates. The next day, the cells were serum- and growth factor-deprived for 24 hr. The cells were imaged in Krebs Henseleit buffer in a 5% CO2 and 60% humidity microscope stage-top incubator. CFP/YFP ratiometric imaging on a 96 well Olympus IX81 automated confocal microscope was performed as previously described (26, 27) over a 20-min time period. Drugs used were: 1µmol/L FEN, 10 nmol/L Ang III, and 1 µmol/L for PD123319 (PD, AT2R antagonist). Both nRPTCs and uRPTCs were used in the cGMP experiments, as this comparison had never been performed. Only nRPTCs were used in the cAMP experiments since uRPTCs don't respond to FEN (lack of adenylyl cyclase stimulation is their defining feature) so synergy with AIII could only be studied in normally responding cells.

PP2A activity assay:
nRPTCs and uRPTCs were grown in 100 mm dishes until 80% confluent, and then serum- and growth factor-starved overnight. Agonists were added [AII (10 nmol/L), AIII (10 nmol/L), FEN (100 nmol/L), Forskolin (FSK, adenylyl cyclase direct agonist, 10 µmol/L)], for 30 min and cells were lysed in phosphate-free lysis buffer with Halt Protease Inhibitor Cocktail (Pierce) according to kit instructions (Millipore, Cat# 17-313). 500 µg of protein were immunoprecipitated using a specific monoclonal antibody against the PP2A catalytic subunit (clone 1D6). Phosphatase activity was measured as the amount of phosphate released from a phospho-peptide (K-R-pT-I-R-R), using malachite green by absorbance at 620 nm in a 96-well plate using a Tecan Ultra microplate reader.
Measurement of phospho-ERK:

nRPTCs and uRPTCs were grown in 6-well plates to 80% confluence and then serum-starved overnight. VEH or FEN was added for 30 min prior to the addition of Ang II (Sigma, 100 nmol/L, 10 min) or antagonists. Angiotensin receptor subtype (AT1R or AT2R) was determined using LOS (1 µmol/L for 10 min), and PD (1 µmol/L for 10 min). Cells were lysed in M-PER with Halt Phosphatase and Protease Inhibitor Cocktail (Pierce). BCA assays were performed to determine protein concentrations and 20 µg of each lysate were added to western loading buffer (without bromophenol blue), boiled, and loaded onto nitrocellulose in a 96-well dot blot apparatus (Bio-Rad).

Immunoreactive monoclonal phospho-ERK 1 and 2 (1:1000, Cell Signaling Inc., Beverly, MA) was detected using an anti-mouse 800 secondary antibody (LI-COR). Immunoreactive rabbit total ERK 1 and 2 (1:1000, Cell Signaling Inc., Beverly, MA) was detected using an anti-rabbit 680 secondary antibody (LI-COR). Membranes were scanned on an Odyssey near infrared imager (LI-COR).

TIRF (total internal reflectance) microscopy:

TIRF microscopy uses an evanescent wave to excite fluorophores in a region less than 200 nm from the surface of the glass holding the specimen. This method allows the visualization of fluorescent molecules bound to and immediately adjacent to the plasma membrane. nRPTCs were passaged onto 25 mm circular polylysine-coated coverslips and placed into 6-well culture plates. Cells were grown to 95% confluence and then serum- and growth factor-starved overnight. Thirty min before agonist addition, monensin (Sigma, 5 µmol/L) was added to cells to increase intracellular sodium. Agonists (FEN, 100 nmol/L or Ang III, 10 nmol/L, or a combination of FEN + Ang III) were added for 30 min before fixing the cells in 4% paraformaldehyde and 1% Triton X-100 for 5 min. They were then washed with 100 mmol/L Tris, followed by PBS. Fixed cells were blocked for 1 hr in 1% BSA and then incubated for 1 hr with a mouse monoclonal antibody to NaKATPase α subunit (Upstate 05-369, 1 µg/ml). Then the cells were washed and incubated for 30 min with Alexa-488-labeled donkey anti-mouse secondary antibody (Invitrogen, 1 µg/ml). The cells were washed again, mounted in PBS and sealed.

Images of the cells were taken with a Hamamatsu Orca R2 camera set at 250 ms for all exposures, using a 60X 1.49 NA objective on a Olympus IX-71 inverted microscope equipped with a “through the objective” cellTIRF illuminator and a 30 mW 488 nm diode laser. The incident angle of illumination was 77.49 and adjusted to 67 nm past the coverslip/water interface using cellTIRF and Metamorph Advanced (version 7, Molecular Devices) software. Image analysis was performed using ImageJ (version 1.36b).

siRNA to GRK4 (G protein-coupled receptor kinase type 4), D1R, and D5R:
The GRK4 target sequence 5’ AATACAAAGAGAAAGTCAA 3’ and scrambled control 5’ AGAAGATAAGAACAATAAC 3’ were chosen from 10 candidate target sequences as the optimum in reducing GRK4 expression by siRNA, as previously published (Gildea et al., 2009). This GRK4 siRNA was then used to test if the failure of the D1R to recruit AT2R to the plasma membrane in uRPTCs was due to a D1R coupling defect caused by GRK4 variants. GRK4 can constitutively phosphorylate and/or modify the D1R, uncoupling it from its effector proteins. 50 nmol/L siRNA or scrambled control were transfected in uRPTCs for 4 hr and the cells were stimulated with FEN 48 hr later, following serum and growth factor starvation.
We also used siRNA to D1R and to D5R in a different set of experiments with nRPTCs in order to determine specifically which receptor was involved in the FEN-mediated AT2R recruitment. 100 nmol/L D1R siRNA (target sequence: 5' CATCTCATCCTCTGTAATA 3'), D1R scrambled control (5'GCATCTTTACACCCTTAT3'), D5R siRNA (target sequence: 5' CCCTTCTTCATCCTTACT3') or D5R scrambled control (5' CTCCATTCCCATCTTTCTAT 3') was transfected for 24 hr into nRPTCs using Lipofectamine 2000 (Invitrogen).

**NaKATPase-mediated sodium efflux assay**

We measured NaKATPase activity as ouabain-sensitive intracellular sodium efflux using an intracellular sodium sensitive dye, as previously described (Gildea et al., 2009). nRPTCs and uRPTCs were cultured in 96-well glass bottom plates coated with collagen, serum- and growth factor-starved overnight, and then labeled with SBFI (Invitrogen, 5 µmol/L). Intracellular sodium was increased by washing cells in potassium-free media twice and then incubating them for 20 minutes in potassium-free media. This was followed by a 10 min addition of 100 nmol/L FEN, 10 nmol/L Ang III, or both (with and without 1 µmol/L PD-123319). Time lapse ratio imaging was conducted using an automated confocal microscope. To initiate sodium efflux, EIPA (5-(N-ethyl-N-isopropyl) amiloride) and potassium were added to the cells to simultaneously block sodium influx and allow the NaKATPase to begin active sodium transport. EIPA is a selective inhibitor of the Na⁺/H⁺ exchanger 3 (NHE3), which is the exclusive sodium hydrogen exchanger in human RPTCs.

**Supplemental References:**

Figure S1. Co-immunoprecipitation and co-localization of D1R and AT2R. In order to determine if D1R and AT2R are found in the same macromolecular protein complex, endogenous receptors in nRPTC (Panels A and B) and stably-transfected receptors in HEK-293 cells (Panel C) were pulled down with rabbit polyclonal antibodies (D1R, Panels B and C, and AT2R, Panel A). Detection was by western blot using a goat polyclonal antibody to AT2R and a rat monoclonal antibody to D1R. D1R and AT2R are found to co-immunoprecipitate in Panels A, B and C, while non-specific rabbit IgG antibody served as a negative control for the immunoprecipitating antibody. The lack of signal with the IgG demonstrated that our washing procedure was adequate in removing non-specific interactions with the Protein A/G Sepharose beads and the non-complementarity determining regions of a rabbit IgG molecule. Experiments were repeated three times with equivalent results. Panel D shows plasma membrane co-localization of the AT2R and D1R. Cell membrane D1R and AT2R were found co-localized in live nRPTC using fluorescently-labelled extracellular epitope-specific antibodies. The D1R polyclonal antibody was labelled with Alexa-555 and the AT2R monoclonal antibody was labelled with Alexa-488. Cells were stimulated with vehicle (VEH) or fenoldopam (FEN, 1 µmol/L, 10 minutes) and corrected FRET (cFRET) was calculated using the FRET Module of Slidebook v 4.2. (N=4, *P<.001 vs. VEH). Images were captured using a 100x 1.4 NA objective.
Figure S2. Relative membrane abundance of AT₁R and AT₂R before and after FEN stimulation. The relative amount of Dylight 649 fluorescently-labeled Angiotensin II (AII, 1 µmol/L) peptide binding to the AT₁R and AT₂R on the plasma membrane of nRPTC and uRPTC was measured with and without FEN (1 µmol/L, 30 min) and the D₁R/D₅R antagonist LE300 (LE, 10 µmol/L, 30 min). All peptide binding in the presence of PD123319 (PD, AT₂R antagonist, 10 µmol/L, 1 hr) represents AT₁R binding. All binding in the presence of losartan (LOS, AT₁R antagonist, 10 µmol/L, 1 hr) represents AT₂R binding. FEN reduced AT₁R binding in both nRPTC and uRPTC and LE blocked this effect. FEN stimulation increased AT₂R binding in nRPTC but not in uRPTC and LE blocked this effect. FEN stimulation switched the AT₁R-dominated surface density to an equal AT₁R and AT₂R plasma membrane density in nRPTC (N=10, *P<0.05 vs. each VEH).
Figure S3. AT1R-stimulated phospho-ERK (pERK1/2) expression is regulated by AT2R and D1R/D5R receptors in the normal D1R/adenylyl cyclase-coupled renal proximal tubule cell (nRPTC) and D1R adenylyl cyclase uncoupled RPTC (uRPTC). The D1R/D5R agonist FEN (1 µmol/L, 30 min) reduced p-ERK1/2 only in nRPTC and not in uRPTC. The AT1R/AT2R agonist angiotensin II (AII, 100 nmol/L, 10 min) stimulates pERK1/2 expression equally in nRPTC and uRPTC. In both nRPTC and uRPTC, AII stimulation is completely blocked by the AT1R antagonist losartan (LOS, 10 µmol/L, 10 min) but not by the AT2R antagonist PD-123319 (PD, 1 µmol/L, 10 min). Treatment with FEN almost completely blocked the AII stimulation of pERK1/2 in nRPTC but only partially blocks the increase in pERK1/2 in uRPTC. Treatment with the AT2R antagonist (PD) partially reversed the FEN-mediated pERK1/2 inhibition in nRPTC but does not have any significant effect in uRPTC. This suggests that part of the inhibitory effect of FEN on AII stimulation of pERK1/2 is mediated by the AT2R (because it is reversed by AT2R antagonist PD), and no such difference is seen in uRPTC (N=4/group in nRPTC and N=6/group in uRPTC, *P<0.05 vs. others, #P<0.05 FEN vs. VEH, &P<0.05 FEN+AII vs. All, ##P<0.05 FEN+All vs FEN+All+LOS, &&P<0.05 FEN+All vs FEN+All+PD, **P<0.05 nRPTC vs uRPTC).
Figure S4. Model depicting how dopaminergic stimulation by fenoldopam (FEN) reduces sodium transport in the human renal proximal tubule cell. Sodium transport is reduced both by the inhibition of the AT1R via D5R, and by the generation of cAMP and cGMP via the D1R and AT2R, respectively. cAMP generation and subsequent activation of PKA inhibits NHE3 and thus reduces sodium influx into the cell. The increase in intracellular cAMP also stimulates PP2A activity, which is necessary for AT2R recruitment to the plasma membrane and resensitization of D1R. In normal cells, plasma membrane AT2R is stimulated by its preferred agonist, Ang III, leading to further increases in PP2A activity. Cells with overactive GRK4 and subsequent D1R coupling defects (uRPTCs) do not respond to FEN stimulation with increased cAMP, activated PP2A or recruited AT2Rs. The D1R and AT2R not only reinforce each other’s unique signaling pathways to increase cAMP and cGMP, respectively, but also increase PP2A activity, which is necessary for full inhibition of NaKATPase and sodium transport.
FIGURE S5

A

FEN  FEN  AngII  LOS  AngII  PD

D₁R  D₂R  AT₁R  AT₂R

AT₂R recruitment

p-ERK

B

FEN  FEN  AngII  LOS

D₁R  D₂R  AT₁R  AT₂R

No AT₂R recruitment

p-ERK
Figure S5. Model depicting how fenoldopam stimulates the recruitment of the AT$_2$R, which in turn inhibits the AT$_1$R’s stimulation of phospho-ERK in nRPTC (Panel A) but not in uRPTC (Panel B) (see Figure S3 for data). Panel A shows a normally coupled cell (nRPTC). Stimulation of D$_1$R and D$_5$R with fenoldopam (FEN) causes inhibition of the AT$_1$R receptor through both D$_1$R and D$_5$R. Additionally, the D$_1$R causes AT$_2$R recruitment to the plasma membrane. Angiotensin II (Ang II) activates the AT$_1$R and subsequently phospho-ERK (pERK1/2) (we used phospho-ERK as a surrogate marker for AT$_1$R stimulation). Ang II alone does not cause AT$_2$R inhibition of AT$_1$R, since AT$_2$R is not recruited to the membrane surface. Treatment of cells with both Ang II and losartan (LOS) blocks AT$_1$R, thus bringing pERK1/2 back to control levels. Ang II and PD have the same effect as Ang II alone. AT$_1$R activates phospho-ERK. PD alone cannot affect the AT$_2$R since it is not on the membrane surface without D$_1$R stimulation.

Treatment of cells with both FEN and Ang II decreases pERK1/2 compared to Ang II alone. D$_1$R and D$_5$R are both activated by fenoldopam, and both work to inhibit the AT$_1$R. The D$_1$R also causes AT$_2$R to be recruited to the membrane surface. Both AT$_1$R and AT$_2$R are then acted on by Ang II. The AT$_2$R inhibits AT$_1$R upon Ang II stimulation, while the AT$_1$R activates pERK1/2. Therefore, treatment with both FEN and Ang II causes D$_1$R, D$_5$R, and AT$_2$R inhibition of AT$_1$R, but also a small increase in pERK1/2 relative to VEH due to AT$_1$R stimulation by Ang II. Treatment with LOS, FEN and Ang II bring pERK1/2 back to control levels. The AT$_1$R is inhibited by LOS. Treatment with PD, FEN and Ang II increases pERK1/2 levels over VEH but less than Ang II alone. AT$_2$R is being inhibited by PD, and cannot inhibit AT$_1$R. However, AT$_1$R is still being inhibited by D$_1$R and D$_5$R.

Panel B shows an uncoupled renal proximal tubule cell (uRPTC). D$_1$R cannot inhibit AT$_1$R and D$_1$R-mediated recruitment of AT$_2$R is defective. Stimulation of D$_1$R and D$_5$R with FEN causes inhibition of the AT$_1$R through D$_5$R only. The D$_1$R cannot inhibit AT$_1$R or cause AT$_2$R recruitment to the plasma membrane. Treatment with Ang II activates pERK1/2 through the AT$_1$R. Treatment of cells with both Ang II and LOS blocks AT$_1$R, thus bringing pERK1/2 back to control levels. Ang II and PD have the same effect as Ang II alone. AT$_1$R activates phospho-ERK. PD does not affect the AT$_2$R since it is not on the membrane surface.

Treatment of cells with both FEN and Ang II increases pERK1/2 levels greater than VEH alone, but not as much as Ang II alone. D$_5$R is activated by FEN and inhibits the AT$_1$R. The D$_1$R does not inhibit AT$_1$R or cause AT$_2$R to be recruited to the membrane surface. AT$_1$R is then acted on by Ang II to activate pERK1/2. AT$_2$R is not recruited to the membrane surface and thus does not inhibit AT$_1$R. Therefore, treatment with both FEN and Ang-II causes D$_5$R inhibition of AT$_1$R, but also a smaller activation of pERK1/2 through the AT$_1$R. Treatment with LOS, FEN and Ang II bring pERK1/2 back to control levels. The AT$_1$R is inhibited by LOS. Treatment with PD, FEN and Ang II increases pERK1/2 to the same extent as uncoupled cells treated with FEN and Ang II. The AT$_1$R is inhibited by D$_5$R, but not D$_1$R, upon FEN stimulation. Ang II acts through AT$_1$R to activate pERK1/2. AT$_2$R is not recruited to the membrane surface and cannot be inhibited by PD.